Modification of aspartate aminotransferase with heavy atoms and with coenzyme analogs

Jane Ann Schmidt
Iowa State University
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MODIFICATION OF ASPARTATE AMINOTRANSFERASE WITH HEAVY ATOMS AND WITH COENZYME ANALOGS

Iowa State University

Ph.D. 1980

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Modification of aspartate aminotransferase with heavy atoms and with coenzyme analogs

by

Jane Ann Schmidt

A Dissertation Submitted to the Graduate Faculty in Partial Fulfillment of The Requirements for the Degree of DOCTOR OF PHILOSOPHY

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Ames, Iowa

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### ABBREVIATIONS

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<td>AAT</td>
<td>aspartate aminotransferase</td>
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<tr>
<td>DNS, dansyl</td>
<td>1-dimethylaminonaphthalene-5-sulfonyl</td>
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<tr>
<td>DTNB</td>
<td>5,5'-dithiobis(2-nitrobenzoate)</td>
</tr>
<tr>
<td>NEM</td>
<td>N-ethylmaleimide</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
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<tr>
<td>PLP</td>
<td>pyridoxal 5'-phosphate</td>
</tr>
<tr>
<td>PLS</td>
<td>pyridoxal 5'-sulfate</td>
</tr>
<tr>
<td>PMP</td>
<td>pyridoxamine 5'-phosphate</td>
</tr>
<tr>
<td>TLC</td>
<td>thin-layer chromatography</td>
</tr>
<tr>
<td>TLE</td>
<td>thin-layer electrophoresis</td>
</tr>
<tr>
<td>TNB</td>
<td>5-thio-2-nitrobenzoate</td>
</tr>
<tr>
<td>TNM</td>
<td>tetranitromethane</td>
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<td>UV</td>
<td>ultraviolet</td>
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\[ kK = 10^3 \text{ Kayser} = 10^3 \text{ cm}^{-1} \]
INTRODUCTION

The work presented in this thesis was undertaken as part of a continuing effort to understand in detail the mechanism of catalysis by the enzyme aspartate aminotransferase. This enzyme is especially well suited for such a study because it contains the cofactor pyridoxal 5'-phosphate (PLP) at each active site. The cofactor forms a covalent bond with the substrate in the active site and is intimately involved in catalysis. Moreover the cofactor acts as a "reporter group," reflecting changes at the active site through changes in its absorption, circular dichroism, and fluorescence spectra.

Pyridoxal phosphate and many of its analogs are quite capable of catalyzing transamination reactions in solution without the apoenzyme. In fact the general theory of pyridoxal-dependent enzymatic reactions as proposed by Metzler et al. (1954) was based on the study of such model reactions and agreed well with the theory of Braunstein and Shemyakin (1953), which was based on the reactions of known pyridoxal-dependent enzymes. From model studies the basic involvement of the cofactor is now well understood.

However, there are fundamental differences between catalysis in model systems and in aspartate aminotransferase. Model systems are strongly stimulated by divalent and trivalent metal ions while the enzyme uses no metal. Pyridoxal in model systems catalyzes a variety of reactions at once, but the enzyme catalyzes the transamination reaction only, and at rates several orders of magnitude greater than those in nonenzymatic systems. Furthermore, a wide variety of amino
acids and pyridoxal analogs are active in nonenzymatic transamination reactions while the enzyme is highly specific for its substrates and for pyridoxal phosphate. These differences must be due to the protein.

The protein imposes constraints on the reaction. The active site has a geometry such that only certain coenzymes and substrates are able to bind. Specific groups on the protein actively participate in catalysis, directing the reaction for transamination by accelerating only the rate of transamination and by inhibiting side reactions.

The goal of this research is to obtain information about the structure of the protein moiety of aspartate aminotransferase and about its interaction with the coenzyme pyridoxal phosphate. Detailed structural information is most easily obtained through X-ray crystallography, and for this reason many researchers have sought to crystallize some form of aspartate aminotransferase. Recently three groups have succeeded in crystallizing the enzyme from different sources (chicken heart cytosol, chicken heart mitochondria, and pig heart cytosol) and have begun the crystallographic structure determination. Of the three, the pig heart cytosolic enzyme is by far the best characterized.

Again, aspartate aminotransferase offers special advantages over most enzymes. The active site is clearly marked by the presence of pyridoxal phosphate. Furthermore, the enzyme forms crystalline complexes with the substrates L-glutamate and L-aspartate and with the pseudo-substrates α-methyaspartate and β-hydroxyaspartate, which form stable transamination intermediates with the enzyme in solution. The polarized light absorption spectra of these crystalline complexes,
which are thought to be true enzyme-substrate complexes, are being studied. The spectra indicate that transamination can occur in the crystals of both the pig heart cytosolic enzyme (Metzler et al., 1978) and the chicken heart mitochondrial enzyme (Eichele et al., 1978). Crystals of the α-methylaspartate and the β-hydroxyaspartate complexes with the pig heart enzyme are known to be isomorphous with those of the native enzyme. It should soon be possible to obtain difference maps which show any changes in the active site which occur during catalysis. It is hoped that these crystalline transamination intermediates will be an invaluable aid in constructing a detailed model of the events that occur at the active site of aspartate aminotransferase during catalysis.

Before detailed difference maps can be calculated, it is necessary to obtain a good high-resolution electron density map of the native enzyme. The first part of this dissertation concerns attempts to prepare crystalline heavy atom derivatives of the enzyme. Taking advantage of the varying reactivities of the five sulfhydryl groups of aspartate aminotransferase, I modified samples of the enzyme at specific sites with mercury-containing reagents, and then tried to crystallize the modified proteins. A variety of sulfhydryl blocking groups and mercury reagents were used in order to find a combination that would give crystals that were isomorphous with crystals of the native enzyme.

Analogs of pyridoxal 5'-phosphate have been used extensively to probe the active site of aspartate aminotransferase and to map
some of the hydrophobic and electronic interactions of the coenzyme with the protein. Two analogs (Figure 1), pyridoxal 5'-sulfate (PLS) and a 5'-trans-carboxyethenyl analog of PLP, provide the basis of the second part of this dissertation. From previous studies, it appears that PLS initially binds to the active site of the apoenzyme as a Schiff base, like pyridoxal phosphate, but then undergoes further reactions to become covalently bound to the protein. The carboxyethenyl analog appears to react similarly. It was proposed that a nucleophilic group of the protein, possibly the same group in both cases, added to the Schiff base in the reactions and that the coenzyme analogs thus may act as crosslinking reagents for two residues at the active site of aspartate aminotransferase. Isolation of modified peptides from these reactions and identification of the amino acid residues involved in the reactions would give information about the geometry of the active site and about the nature of the reactions of these coenzyme analogs with the protein. In addition, it may be possible to locate another amino acid residue at the active site, in close proximity to the active site lysine, and this would help in tracing the polypeptide chain in the X-ray structure determination.

Analogs of pyridoxal phosphate, including PLS and the carboxyethenyl analog, have in the past been assumed to bind to the active site of aspartate aminotransferase on the basis of their ability to inhibit the binding of pyridoxal phosphate. It is important that in at least one case a peptide modified by one of these analogs be isolated and identified as the peptide that contains the active site lysine in order
Figure 1  Structures of coenzymes used in this study. I. pyridoxal 5'-phosphate. II. pyridoxal 5'-sulfate. III. 5'-trans-carboxyethenyl analog.
to demonstrate that this assumption is valid. This is another goal of this research.

For this purpose I digested apoAAT modified by PLS or the carboxy-ethenyl analog with pepsin to obtain small peptides that contained the more stable low pH forms of the chromophoric products. At low pH, the PLS product has a brilliant blue-white fluorescence and a characteristic absorption spectrum, so that the product could easily be detected and measured quantitatively throughout the purification procedure. The peptides were separated by gel chromatography and thin-layer electrophoresis. The peptides that contained the PLS product were used to identify the amino acids involved in the reaction of PLS and apo-aminotransferase and to study the chemistry of the reaction.
LITERATURE REVIEW

Aspartate Aminotransferase

Aspartate aminotransferase (AAT) from pig heart cytosol was the first vitamin B$_6$-dependent enzyme to be obtained in virtually pure state (Jenkins et al., 1959), and has become the most studied and therefore the most thoroughly characterized enzyme of this class. The properties of AAT have been the subject of several reviews (Ivanov and Karpeisky, 1969; Braunstein, 1970; Turano et al., 1970; Severin and Gulyaev, 1976) and are best summarized by Braunstein (1973).

AAT is an apparently symmetric dimer with a mass of 93,147 daltons. The active sites function independently (Schlegel and Christen, 1974, 1978; Boettcher and Martinez-Carrion, 1975a, 1976; Schlegel et al., 1977). However, there are strong interactions between subunits so that the binding of PLP to one subunit of a dimer of apoenzyme elicits a conformational change in the other subunit (Schlegel and Christen, 1974; Arrio-Dupont and Cournil, 1975).

There is disagreement as to whether the protomers dissociate on dilution. Cournil and coworkers (1975) reported a dissociation constant of 1.8 x 10$^{-7}$ M while Melander (1975) estimated $K_d$ to be 10-fold lower. On the other hand Feliss and Martinez-Carrion (1970) found no evidence for dissociation of the subunits on dilution to 10$^{-10}$ M. The subunits can be dissociated by denaturation (Feliss and Martinez-Carrion, 1970) or by succinylation of the protein (Polyanovsky, 1965).

Each subunit consists of a single polypeptide of 412 residues,
whose sequence has been determined independently by two groups (Ovchinnikov et al., 1973; Doonan et al., 1975). One molecule of the cofactor pyridoxal 5'-phosphate is bound as a Schiff base to lysine-258 of each subunit (Fischer et al., 1962; Hughes et al., 1962). The bound coenzyme exists in several spectrophotometrically distinguishable forms. The enzymatically active form has an absorption maximum at 363 nm (27.4 kK). This form is related to another form absorbing at 430 nm (23.3 kK) by a single pK_a that varies between 5.4 and 6.3, depending on the identity and concentration of the buffer anion (Bergami et al., 1968). These two forms are related through the addition of a proton to the nitrogen atom of the Schiff base (Jenkins et al., 1959; Cheng et al., 1971). In addition, there is an inactive form of the coenzyme with a pH-independent absorption maximum at 340 nm (29.4 kK). The nature of the enzyme-coenzyme bond in this form is not understood (Martinez-Carrion et al., 1967b).

Starch gel electrophoresis of AAT as it was originally isolated gives at least three distinct bands (Martinez-Carrion et al., 1967b; Banks et al., 1968). The three forms of the enzyme present in the major bands are known as the α, β, and γ subforms, in the order of their increasing anodic mobility on starch gel electrophoresis. The α subform contains two molecules of PLP per dimer, both bound in the active mode with a pH-dependent absorption maximum at 430 or 363 nm, as described above. The α form is the enzymatically most active subform. The γ subform is the least active with its coenzyme bound predominately in the inactive mode, absorbing at 340 nm. The β
subform has an intermediate activity and contains approximately one-half actively bound and one-half inactively bound PLP. Each dimer of the β subform has one active and one inactive monomer (Martinez-Carrion et al., 1967b; Evans and Holbrook, 1974). Upon aging, the purified α and β subforms lose activity slowly as the coenzyme becomes bound in the inactive mode. However, the electrophoretic mobilities do not change (Martinez-Carrion et al., 1967b). Inactive subunits can be partially reactivated by denaturation and renaturation. It has been proposed that denaturation may break the bonds responsible for the inactive mode of PLP binding. An alternative explanation is that the inactive mode is favored as a thermodynamically more stable conformation which is formed by a slow transition from a less stable, but active, conformation; the latter is favored kinetically during renaturation (Giartosio et al., 1973). The α and β subforms differ by a single charge in the pH range 5.6 to 8.0, possibly due to the hydrolysis of an amide group (John and Jones, 1974). Consistent with this, Williams and John (1979) have observed the production of ammonia and generation of negative charge in AAT in vitro consistent with the deamination of two glutamine or asparagin residues per dimer.

The mechanism of transamination by AAT is shown in the half-reaction in Figure 2 (Braunstein, 1973). The enzyme active site contains the coenzyme PLP bound as a Schiff base to a lysine residue (I). The first step in the reaction sequence is the binding of the substrate amino acid to form a Michaelis complex (II). The amino
Figure 2  Half transamination reaction, as catalyzed by aspartate aminotransferase. From Braunstein (1973).
acid substrate then presumably condenses with the internal Schiff base to form a transient tetrahedral adduct (III). This form is too short-lived to be observed spectrophotometrically. The lysine side chain is then released to complete the transimination from the enzyme-coenzyme Schiff base to the substrate-coenzyme Schiff base (IV).

The third step in the reaction sequence is a prototropic aldimeine-ketimine rearrangement. A basic group of the protein removes the α-proton of the amino acid substrate to form a transient quinonoid structure (VI). The abstracted proton is then transferred to the 4'-carbon of the coenzyme in what appears to be the rate-limiting step of the reaction (Harruff and Jenkins, 1978). Finally, the ketimine (VII) is hydrolyzed to a keto acid and pyridoxamine 5'-phosphate (VIII).

The pseudosubstrate α-methylaspartate forms a Schiff base with the coenzyme at the enzyme active site analogous to structure IV in Figure 2, but because there is no α-proton the reaction with this amino acid cannot proceed further. Erythro-β-hydroxyaspartate forms a stable quinonoid structure (VII) with the enzyme with a strong absorbance at 490 nm (20.3kK). This absorbance is seen only faintly with the substrate pair aspartate and oxaloacetate. Difluorooxaloacetate reacts slowly with the PMP form of the enzyme and has been suggested as a probe to study the aminic forms of AAT (Briley et al., 1977a,b).
Reactive amino acid residues

Several amino acid residues of AAT can be modified selectively with varying effects on the activity of the enzyme. Those residues whose modification leads to the inactivation of the enzyme are suspected of being located in the active site, and in some cases have been assigned a role in the catalytic mechanism. The evidence of these chemical modification studies is indirect, and thus is not always reliable. Conclusions drawn from such evidence are often controversial.

Lysine In the native holoenzyme the coenzyme is bound to the protein in a Schiff base linkage to the ε-amino group of lysine residue 258 in the polypeptide chain (Hughes et al., 1962; Ovchinnikov, et al., 1973). This Schiff base not only forms a covalent link between the enzyme and coenzyme, but it makes the 4'-carbon of the coenzyme much more susceptible to nucleophilic attack than would be the free aldehyde (Cordes and Jencks, 1962).

The protonated Schiff base forms one of the binding sites for dicarboxylic acid inhibitors of the enzyme (Martinez-Carrion et al., 1973). Lysine-258 is alkylated by the amino acids β-chloro-L-alanine, L-propargylglycine, and vinylglycine, causing the complete inactivation of the enzyme (Morino and Okamato, 1973; Tanase, 1977; Gehring et al., 1977b). The unprotonated ε-amino group of this lysine residue has been proposed as the group that accepts a proton from the α-carbon of the amino acid substrate and transfers it to the 4'-carbon of the coenzyme during catalysis (Ayling et al., 1968; Ivanov and Karpeisky, 1969;
Histidine both chemical modification (Turano et al., 1966; Severin et al., 1972; Kovaleva and Severin, 1972) and photoinactivation studies (Martinez-Carrion et al., 1967a; Martinez-Carrion et al., 1970; Peterson and Martinez-Carrion, 1970; Burridge and Churchich, 1970; Yamasaki et al., 1975) have been used to detect at least one reactive histidine residue in AAT. A histidine side chain was modified by the bifunctional reagent 3-bromopropionyl chloride which reacted first with a lysine residue, presumably lysine-258 (Turano et al., 1966, 1970). A histidine residue may also have been alkylated in the reaction of the enzyme with the substrate analog threo-α-cycloglutamic acid, but the product was unstable and could not be characterized (Kovaleva and Severin, 1972).

The critical histidine is not involved in coenzyme binding (Martinez-Carrion et al., 1970) but there is disagreement about its function. Because the pH-dependence of the rate of the cycloglutamate reaction had an inflection point at pH 6.7, Severin et al. (1972) proposed that the imidazole of the histidine binds the α-carboxyl group of the substrate. Martinez-Carrion et al. (1973) proposed that the histidine binds a carboxyl group of the dicarboxylic acid inhibitors but not of substrates because the group that is involved in substrate binding has no pH-dependence in the range of pH 5-9. Photooxidized enzyme can bind substrates and form an aldimine complex. However it cannot catalyze α-proton exchange of an amino acid substrate, and it does not show an absorption at 490 nm with hydroxy-
aspartate due to the quinonoid intermediate. These observations lead to the proposal that the functional histidine residue may be the group that transfers the proton from the \( \alpha \)-carbon of the amino acid to the 4'-carbon of the coenzyme (Ayling et al., 1968; Peterson and Martinez-Carrion, 1970; Cheng and Martinez-Carrion, 1972).

**Tyrosine** Christen and Riordan (1970) found that while tetranitromethane (TNM) did not inactivate AAT in either the PLP or PMP form, in the presence of the substrate pair glutamate and \( \alpha \)-ketoglutarate, TNM inactivated the enzyme with the nitration of one tyrosine residue. During the course of the reaction the enzyme was converted to the PMP form. Although \( \alpha \)-ketoglutarate could bind to the modified enzyme and could form a Schiff base with the PMP, the coenzyme could not be reconverted to PLP (Bocharov et al., 1973). In the presence of either substrate alone, of the substrate analog \( \alpha \)-methylaspartate, or of the competitive inhibitors maleate, glutarate, or phthalate, TNM caused only slight inactivation. Inactivation, therefore, seemed to reflect a short-term change in the environment of a tyrosine residue, probably during or after the transition from the aldimine to the ketimine (Christen and Riordan, 1970). This modification was termed "syn-catalytic" since it appeared to be synchronous with catalysis. Polyanovsky et al. (1972) isolated a peptide containing the nitrotyrosine residue and identified the reactive residue as tyrosine-40.

Later results showed that TNM modified cysteine as well as tyrosine residues of the enzyme. Thus, disagreement arose about whether the modification of tyrosine or the modification of cysteine actually
caused the inactivation. Turano et al. (1971) found that the inactivation of AAT correlated better with the modification of tyrosine than with the modification of cysteine. On the other hand, Birchmeier et al. (1973b) found that the nitration of tyrosine was not as specific as the oxidation of cysteine since no more than one tyrosine per monomer was modified but only half the nitrotyrosine appeared in any one peptide resulting from cyanogen bromide cleavage of the enzyme. They concluded that the nitration of tyrosine was a secondary reaction facilitated by the prior oxidation of cysteine. Bocharov et al. (1973) selectively modified either tyrosine-40 or cysteine-390 and found that complete inactivation occurred only with modification of both residues.

The bifunctional reagent difluorodinitrobenzene crosslinked the active site lysine and tyrosine-70 in apoAAT, establishing the presence of a tyrosine in the active site about 0.5 nm from lysine-258 (Turano et al., 1970; Deev et al., 1978).

Tyrosine or tryptophan Based on studies of the circular dichroism of the enzyme, Ivanov and Karpeisky (1960) proposed that the active site tyrosine is hydrogen-bonded to the pyridine N of the coenzyme. The circular dichroic spectra of the PMP enzyme and of the PLP enzyme in its high pH and low pH forms all showed a negative band 295 to 300 nm which belonged to the enzyme but was absent in the spectrum of the apoenzyme. These authors maintained that only a tyrosine residue can account for the position and shape of this band. When PLP N-oxide, an analog of PLP that cannot form a hydrogen bond to
the ring N, was added to apoAAT, the CD spectrum again showed a negative band at 300 nm and a new positive band appeared at 275 nm, close to the absorption maximum of an unionized tyrosine. This was in agreement with findings that apoAAT has more tyrosines available to TNM than holoAAT, and that apoAAT was completely inactivated by TNM due to the failure of nitrated apoenzyme to recombine with PLP (Turano et al., 1971). However, because the circular dichroism band at 300 nm corresponds to vibronic fine structure that appeared when PLP, PMP, or coenzyme analogs were bound to apoAAT, Yang et al. (1975) ascribed this band to an alteration in the environment of a tryptophan residue rather than tyrosine, whose absorption spectrum lacks fine structure. Thus, while there probably is a tyrosine residue in the active site, there is uncertainty about its exact position and its role.

Cysteine AAT has five cysteine and no cystine residues in each monomer (Ovchinnikov et al., 1973; Doonan et al., 1975). The cysteines differ greatly in their reactivities toward sulfhydryl reagents and in the effects of their modifications on the activity of the enzyme (Cournil and Arrio-Dupont, 1971; Stankewicz et al., 1971; Birchmeier et al., 1973a,b).

Cysteine-45 is the most highly reactive sulfhydryl group of the five and reacts with 5,5'-dithiobis(2-nitrobenzoate) (DTNB) at a rate of 800 M⁻¹s⁻¹ at pH 7.5. The rate of modification is unaffected by the presence of glutamate, α-ketoglutarate, or both in the reaction mixture. Modification of cysteine-45 with N-ethylmaleimide (NEM) or with TNM
causes a slight increase and decrease, respectively, in the enzymatic activity. Reaction of cysteine-45 with other sulfhydryl reagents has no effect on the activity (Zufarova et al., 1973; Wilson et al., 1974). Cysteine-45 can be selectively modified by NEM, DTNB, TNM (Wilson et al., 1974), or maleate (Polyanovsky et al., 1973a).

Cysteine-82 is also highly reactive, but reacts with DTNB about 20 times slower than does cysteine-45. The rate of reaction is 26 M$^{-1}$ s$^{-1}$ at pH 7.5, and, like that for cysteine-45 it is unaffected by the presence of substrates (Zufarova, et al., 1973; Wilson et al., 1974).

Fluorescence properties of mixed disulfides of N-dansylcysteine with cysteine-45 and cysteine-82 indicated a polar environment such as that of the exterior of the enzyme (Wilson et al., 1974). Electron spin resonance studies of spin labels attached to cysteine-45 and cysteine-82 also indicated that the groups are located on the exterior of the protein 2.0-2.3 nm apart and at a distance of about 2.5 nm from the active site. On interaction of the spin labeled enzyme with substrates and with quasisubstrates, the ESR spectrum of the spin label on cysteine-45 changed reversibly, indicating a conformational change in this region of the enzyme (Polyanovsky et al., 1973b; Polyanovsky and Misharin, 1974).

Two more sulfhydryl groups, cysteine-191 and cysteine-252, are unreactive, except under denaturing conditions (Birchmeier et al., 1973a; Zufarova et al., 1973).

The fifth cysteine, residue 390, is only slightly reactive in the native enzyme. Its rate of reaction with DTNB is $2 \times 10^{-2} \text{ M}^{-1} \text{ min}^{-1}$. 
five orders of magnitude less than that of cysteine-45 and cysteine-82. However, like tyrosine-40, it becomes more reactive in the presence of a substrate pair and thus is also syncatalytically modifiable. In the presence of the substrate pair glutamate and α-ketoglutarate, this residue becomes twenty times more reactive and its rate of modification with DTNB is $4.0 \times 10^{-1} M^{-1} \text{min}^{-1}$ (Cournil and Arrio-Dupont, 1971; Birchmeier and Christen, 1971; Birchmeier et al., 1972; Torchinsky et al., 1972; Birchmeier et al., 1973a). Since cysteine-390 is less reactive in the presence of either substrate alone, α-methylaspartate, or β-hydroxyaspartate, it is probably most reactive in the ketimine intermediate (Birchmeier and Christen, 1971; Birchmeier et al., 1973a). Modification of cysteine-390 with a variety of sulfhydryl reagents such as NEM, DTNB, \textit{p}-chloromercuribenzoate, TNM, sulfite, glutathione, mercaptoethanol, methylmercaptan, or 3-bromopyruvate severely impaired the activity of the enzyme. However, the enzyme was not completely inactivated and could still be quantitatively converted between the PLP and PMP forms by the addition of the appropriate substrate. Furthermore, when cysteine-390 was modified with cyanide, which leaves a small uncharged substituent, the enzyme retained 60% of its initial activity. It therefore seems that cysteine-390 has no essential role in catalysis (Birchmeier et al., 1973a; Birchmeier and Christen, 1974). However, the residue is located very near to the active site. Difluorodinitrobenzene crosslinked lysine-258 and cysteine-390 in apoAAT, indicating that the two residues are about 0.5 nm apart (Deev et al., 1978).

The special reactivity of cysteine-390 has been utilized in a
variety of ways to explore the environment of this residue. From the fluorescence properties of a mixed disulfide of cysteine-390 with N-dansylcysteine, Wilson et al. (1974) concluded that the residue was located in a rather hydrophobic environment and near a tryptophan residue. Boettcher and Martinez-Carrion (1975b) modified cysteine-390 with $^{13}$C-CN and recorded the $^{13}$C nuclear magnetic resonance spectrum at different pH's and with the addition of substrates or substrate analogs. Since they found no change in the chemical shift of the probe, there is apparently no nearby group that ionizes under those conditions. Boettcher and Martinez-Carrion (1978) attached a spin label, 4-maleimido-2,2,6,6-tetramethylpiperidinoxyl, to cysteine-390 and measured its effects on the nuclear magnetic resonance properties of various ligands bound at the active site of the enzyme. In this way they were able to map the distances between the spin probe and nuclei of the ligands. They concluded that the nitroxide ring was located above or below the plane of the coenzyme ring, 0.8-0.9 nm away, and that the $\alpha$-methyl and $\beta$-methylene protons of the substrate analog $\alpha$-methylaspartate were located in the same plane as the coenzyme ring, about 0.9 nm away. $^{19}$F nuclear magnetic resonance studies of 1,1,1-trifluoroactonylated cysteine-390 was used to detect conformational changes in the region of cysteine-390 when substrate was bound to the active site. The line width of the NMR signal decreased, indicating that more motion was allowed in the region of the probe (Critz and Martinez-Carrion, 1977a,b).

**Arginine** The arginine-specific reagents 2,3-butanedione,
phenylglyoxal, and 1,2-cyclohexanedione reversibly inactivated AAT by modifying approximately two arginine residues per subunit (Riordan and Scandurra, 1975; Gilbert and O'Leary, 1975). The essential arginines did not appear to bind the coenzyme phosphate, as had been expected, because the enzyme that was resolved either before or after phenylglyoxal modification rebound PLP with no restoration of activity (Riordan and Scandurra, 1975). The arginine appeared to form the binding site for the ω-carboxyl of the substrate since incubation of the cyclohexanedione-modified enzyme with aspartate, cysteine sulfinate, or alanine caused a decrease in the absorbance of the enzyme at 430 nm and an increase at 330 nm due to a slow transamination. All three substrates reacted at about the same rate, although alanine is a much poorer substrate for the native enzyme than the others are (Gilbert and O'Leary, 1975).

**Threonine** There is some indication of a threonine residue in the active site of AAT. N-Phosphopyridoxyl-L-glutamic acid reacted at the active site of apoAAT to inhibit the enzyme irreversibly. Although this analog of the coenzyme-substrate complex is itself not an alkylating agent, the reaction involved transfer of the phosphate group to a threonine residue of the protein with the participation of nucleophilic group(s) of the protein. This threonine residue may be involved in the catalytic mechanism (Khomutov et al., 1969; Braunstein, 1973).

**Carboxyl groups** Although blockage of 45% of the carboxyl groups of the enzyme greatly reduced the activity of the enzyme, Bossa et al. (1971) found no evidence of the presence of a carboxyl group at the
active center of the enzyme.

Crystallization

In order to understand the mechanism of catalysis by aspartate aminotransferase, a picture of its structure is needed. Some structural information has been deduced indirectly from the modification experiments discussed above, but a complete picture can be had only from X-ray crystallography. Therefore, many attempts have been made to crystallize the enzyme. Bertland and Kaplan (1968, 1970) crystallized both the soluble and mitochondrial forms of AAT from chicken heart as part of their isolation procedures. They added ammonium sulfate to a solution of either isozyme until a faint turbidity appeared. This precipitate was removed by centrifugation, and the solution was allowed to stand until crystals appeared, usually in a day or two. The crystals of neither enzyme were of the size or quality suitable for crystallography.

Koeppe et al. (1975) reported crystallization of pig heart cytosolic AAT by a pulsed diffusion technique which alternately precipitated and redissolved the enzyme by dialysis against concentrated and dilute solutions of 2-methyl-2,4-pentanediol. The crystals obtained this way were not large enough for crystallography.

Recently, three research groups have crystallized aspartate aminotransferase from different sources and have begun the crystallographic structure determination. Borisov et al. (1977) crystallized chicken heart cytosolic AAT as the α-methylaspartate complex by salting out with ammonium sulfate and cesium chloride in the presence
of a semipolar solvent. They have calculated an electron density map of the enzyme at 5 Å resolution and have located the active site by means of a difference map of the crystalline apoenzyme (Borisov et al., 1978). They have also calculated difference maps of the PLP-enzyme, PMP-enzyme, and the enzyme-isonicotinic acid complex against the α-methylaspartate complex. These difference maps showed large positive and negative peaks, indicating that those forms have a different conformation than the apoenzyme and the α-methylaspartate complex. More recently they have calculated an electron density map at 3.5 Å, from which they were able to trace most of the polypeptide chain (Borisov et al., 1979).

Arnone et al. (1977) crystallized pig heart cytosolic AAT by vapor diffusion against a solution of polyethylene glycol 6000 using the "sandwich box" technique of McPherson (1976). They have calculated an electron density map at 2.7 Å resolution and have located the coenzyme and substrate binding sites by means of difference maps of the apoenzyme and of crystalline complexes with erythro-β-hydroxyaspartate and α-methylaspartate (Metzler et al., 1979).

Gehringer et al. (1977a) have crystallized mitochondrial AAT from chicken heart by vapor diffusion against a solution of polyethylene glycol using the "hanging drop" method. They have calculated an electron density map at 3.2 Å resolution from which they could trace the entire polypeptide chain in both subunits of the dimeric enzyme (Eichele et al., 1979a). More recently they constructed a model of the active site of the enzyme based on a map at 2.8 Å resolution.
(Ford et al., 1980). Jansonius and coworkers are also collecting data on mitochondrial AAT from pig hearts, which forms crystals isomorphous with crystals of the enzyme from chicken heart mitochondria (Eichele et al., 1979b).

Absorption spectra of crystalline pig heart cytosolic AAT (Metzler et al., 1978) and of chicken heart mitochondrial AAT (Eichele et al., 1978) are being studied. Both enzymes are completely interconverted between the PLP and PMP forms by diffusion of appropriate substrates into the crystals. Both crystalline enzymes also show the absorption band of a quinonoid intermediate with β-hydroxyaspartate. Polarized light absorption spectra indicate that the coenzyme ring rotates during catalysis (Metzler et al., 1978; Jansonius et al., 1979), although not along the C-2 and C-5 axis as suggested by Ivanov and Karpeisky (1969) from circular dichroic spectra.

Coenzyme Analogs

The presence of the covalently-bound cofactor pyridoxal 5'-phosphate in the active site of aspartate aminotransferase gives an especially useful tool for studying the active site of the enzyme. A large number of analogs of PLP have been synthesized in order to study the interactions of the coenzyme with the protein. The binding of these analogs to apoAAT is followed by absorption and circular dichroism spectroscopy, and any complexes that are formed are tested for enzymatic activity and for the inhibition of PLP binding.

In such studies, it was found that, although N-methylpyridoxal phosphate and PLP N-oxide bound to the apoenzyme, a free ring nitrogen
is essential for catalysis. Similarly, analogs with an absent or modified 3'-hydroxyl group bind to apoAAT but are inactive. The 4'-formyl group is essential for tight binding (formation of the enzyme-coenzyme Schiff base) and for enzymatic activity, although the 4'-ethynyl analog of PLP appears to form a Schiff base by addition of an amino group of the protein to the ethynyl group of the coenzyme and rearrangement of the resulting enamine (Yang et al., 1975). The apoenzyme seems to have considerable spatial tolerance for substituents at the 2 and 6 positions of the coenzyme ring. 2-Nor-PLP, 2'-methyl-PLP, 2',2'-dimethyl-PLP, 6-methyl-PLP, and 2-nor-6-methyl-PLP all bind stoichiometrically to the active site and produce complexes with enzymatic activities comparable to that of holoAAT (Braunstein, 1973; Fonda, 1971; Furbish et al., 1969; Yang et al., 1975).

The 5'-phosphate ester of PLP appears to be necessary for firm binding of the coenzyme to the enzyme and for effective catalysis, since most analogs modified at this position have little or no catalytic activity (Braunstein, 1973; Fonda, 1971; Furbish et al., 1969). Analogs lacking the phosphate group do not inhibit apoAAT, even if they have alkylating groups at the 4'-position (Severin and Gulyaev, 1976). Some analogs such as the 5'-trans-carboxyethenyl analog (Miura and Metzler, 1976) and pyridoxal 5'-sulfate (Yang et al., 1974) inactivate apoAAT, becoming covalently bound. Mechanisms for these reactions have been proposed based on studies of model reactions.
**Pyridoxal 5'-sulfate**

Pyridoxal 5'-sulfate (PLS) is structurally quite similar to PLP since the sulfate and phosphate groups are both tetrahedral. The sulfur-oxygen bonds are only about 0.06 Å shorter than the phosphorus-oxygen bonds, and the bond angles are almost identical. Chemically the two differ in that the esterified sulfate group lacks the second ionizable proton of the esterified phosphate.

In spite of the structural similarities, PLS can act as a coenzyme with few of the PLP-requiring enzymes with which it has been tested. It does not bind to the apoenzyme of L-aspartate-β-decarboxylase (Tate and Meister, 1969). It binds to, but does not activate, mouse brain glutamate decarboxylase (Matsuda and Makino, 1961), *E. coli* glutamate decarboxylase (Likos, 1977), and D-serine dehydratase (Groman et al., 1972). PLS does not form a stable complex with serine hydroxymethylase from rabbit liver, but excess PLS inhibits PLP binding to the apoenzyme (Schirch and Schnackerz, 1978). In contrast, preliminary studies with the enzyme from sheep liver indicate that the apoenzyme can be partially reactivated with PLS (J.D. Cook and L. Davis, Department of Chemistry, University of Iowa, unpublished results). PLS induces the reassociation of the subunits of apophosphorylase, but the complex is inactive (Shaltiel et al., 1969). It binds very poorly to apotryptophanase, but the complex is slightly active. Arginine decarboxylase can be reconstituted with PLS and regains 23% of the activity of the enzyme reconstituted with PLP (Groman et al., 1972).

The interaction of PLS with the apoenzyme of AAT has been studied
in detail (Yang et al., 1974). PLS binds to the apoenzyme initially forming a Schiff base, but undergoes further reactions to become covalently bound to the protein. The complex has no measurable enzymatic activity.

Pyridoxal sulfate reacts with a variety of amines and amino acids with nucleophilic substituents in the $\alpha$-position in solution at pH 9. The spectral changes observed during these reactions were similar to those seen in the enzymatic reaction, so the reaction of PLS and cysteine was studied as a model for the enzymatic reaction. Based on the known reaction of pyridoxal phosphate and cysteine, the determination of free sulfate in the reaction mixture, and proton nmr, the reaction mechanism in Figure 3 was proposed. Pyridoxal sulfate (I) and cysteine react initially to form a Schiff base (II) and then a thiazolidine (III), in the same manner as PLP and cysteine react (Abbott and Martel, 1970). However, the PLS-cysteine thiazolidine reacts further, losing a proton from the 4'-carbon of the coenzyme and eliminating sulfate to form a quinonoid intermediate (IV). This undergoes a prototropic shift to form a cyclic substituted Schiff base (V), which is the final product. The nmr spectra of the reaction mixture recorded at various times during the reaction substantiate this scheme, but also indicate that the reaction is not completely clean. Several spurious nmr peaks appear during the reaction and are present in the spectrum of the final product.

It was proposed that a nucleophilic group at the active site of aspartate aminotransferase reacts with the Schiff base of PLS and the active center lysine in a similar reaction.
Figure 3  Proposed mechanism of the reaction of pyridoxal 5'-sulfate with cysteine, a model for the reaction of pyridoxal sulfate with aspartate aminotransferase (Yang, et al., 1974).
MATERIALS

Enzymes

The $\alpha$ subform of pig heart cytoplasmic aspartate aminotransferase was isolated in this laboratory by the method of Jenkins et al. (1959) as modified by Martinez-Carrion et al. (1967b, Method A). The apoenzyme was prepared by the method of Scardi et al. (1963) as modified by Furbish et al. (1969).

Pepsin, trypsin, and chymotrypsin were purchased from Worthington Biochemical Corp. Aminopeptidase M was purchased from the Pierce Chemical Co.

Pyridoxal Phosphate Analogs

Pyridoxal 5'-sulfate was prepared in this laboratory by Dr. Allen Cahill by the procedure of Yang et al. (1974). The 5'-trans-carboxyethenyl analog of pyridoxal phosphate was synthesized by Dr. Retsu Miura (Miura and Metzler, 1976).

Other Chemicals

N-ethylmaleimide, $p$-chloromercuribenzoic acid, iodoacetamide, 5,5'-dithiobis(2-nitrobenzoic acid), 1-dimethylaminonaphthalene-5-sulfonyl chloride (Dansyl chloride), dansyl-glutamate, dansyl-serine, dansyl-valine, dansyl-isoleucine, dansyl-arginine, $\varepsilon$-dansyl-lysine, $\alpha$,$\varepsilon$-bisdansyl-lysine, pyridoxal 5'-phosphate, and pyridoxamine 5'-phosphate were purchased from Sigma Chemical Co. Methylmercuric chloride was obtained from Research Organic/Inorganic Chemicals. The tetrafluoroborate salt of diazotized sulfanilate was purchased
from Calbiochem-Behring Corp.

All other chemicals were purchased from commercial sources and were of reagent grade or better.

Dansyl-alanine, dansyl-glycine, dansyl-phenylalanine, O-dansyl-tyrosine, and N,O-bisdansyltyrosine were prepared from the corresponding amino acids by the procedure of Gray (1972).
METHODS

The concentration of aspartate aminotransferase was determined from the absorption of the solution at 35.8 kK (280 nm), using a molar absorptivity of $6.55 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$ for the holoenzyme and a molar absorptivity of $6.36 \times 10^4$ for the apoenzyme (Furbish et al., 1969). The concentrations refer to the monomer.

Determination of Free Sulfhydryl Groups

Free sulfhydryl groups of AAT were determined by titration with 5,5'-dithiobis(2-nitrobenzoate), DTNB, as described by Birchmeier et al. (1973a), using $\varepsilon_{412 \text{nm}} = 13600 \text{ M}^{-1}\text{cm}^{-1}$ for 5-thio-2-nitrobenzoate (Ellman, 1959). Protein with approximately 5 nmol sulfhydryl groups was added to 1.0 mL 1% sodium dodecyl sulfate in 0.1 M sodium phosphate, pH 7.5, in a semimicro cuvette. After about 5 min, when the absorbance at 412 nm had reached a constant value, 10 μL of 40 mM DTNB in 0.1 M phosphate was added. The increase in the absorbance at 412 nm was followed on a Gilford-Beckman DU spectrophotometer until it reached a maximum value in about 5 min. A second cuvette containing no protein was used to correct for the absorbance of DTNB.

Modification of Sulfhydryl Groups

Hereafter, "phosphate buffer" refers to 0.10 M sodium phosphate, pH 7.5, and "acetate buffer" refers to 0.04 M sodium acetate, pH 5.4.

Sulfhydryl group modifications were performed in phosphate buffer at room temperature, unless otherwise specified. Modified proteins were separated from excess reagents and low molecular weight products.
on a column of Sephadex G25 (1.1 x 35 cm) equilibrated with phosphate buffer if the protein was to be modified further, or with acetate buffer if it was to be crystallized directly.

Cys45 and Cys82 of AAT were modified with N-ethylmaleimide (NEM) by the procedure of Birchmeier et al. (1973a). AAT (2 x 10^{-4} M monomer) was allowed to react with 2 mM NEM for 1 h. at room temperature.

Modification of Cys45 and Cys82 with cyanide was accomplished in two steps (Birchmeier et al., 1973a). AAT (10^{-4} M) was first reacted with 0.4 mM DTNB to form a mixed disulfide between cysteine and 5-thio-2-nitrobenzoate (TNB). The bound TNB was then displaced by adding solid KCN to a concentration of 10 mM. Both reactions could be followed spectrophotometrically by the increase in absorbance at 412 nm due to the release of TNB^- . The first reaction was complete within 10 min; the second step took about 30 min. The modified protein was dialyzed overnight against acetate buffer to remove cyanide which had apparently formed an adduct with the coenzyme.

AAT blocked at Cys45 with cyanide was prepared by first forming the TNB derivative by the procedure of Wilson et al. (1974) and then displacing the TNB with cyanide. AAT (10^{-4} M) mixed with an equivalent amount of DTNB in a spectrophotometer cell. The reaction was followed by the increase in absorbance at 412 nm until approximately 0.96 equivalent of TNB^- was released. Bound TNB was displaced by cyanide as described above.

Cys45 and Cys82 were modified with iodoacetamide by the procedure of Critz and Martinez-Carrion (1977a). AAT (2 x 10^{-4} M monomer) was
allowed to react with 0.4 M iodoacetamide in 0.1 M potassium phosphate, pH 6.0, at 4° for 24 h in the dark.

**Syncatalytic modifications**

Cys390 of AAT blocked at Cys45 and Cys82 by NEM was modified syncatalytically in phosphate buffer containing 70 mM L-glutamate, 2 mM α-ketoglutarate, 1 mM PLP, and 1 mM PMP (Birchmeier et al., 1973a). The enzyme was reacted with either 0.11 mM p-chloromercuribenzoate or 0.11 mM methylmercuric chloride for 24 h at 4°. Excess reagents, substrates, and coenzymes were removed on a column of Sephadex G25 (1.1 x 35 cm) equilibrated with acetate buffer.

To prepare the cyanide derivative of Cys390, NEM-blocked enzyme was reacted with 0.12 mM DTNB for 24 h under the conditions for syncatalytic modification outlined above (Birchmeier et al., 1973a). After the excess reagents were removed, solid KCN was added to about 10 mM to displace the bound TNB.

To determine whether or not methylmercuric chloride binds to AAT modified at Cys45 and Cys82 under the conditions for crystallization, iodoacetamide-modified AAT (34.7 mg/mL) was incubated with 1.05 eq of methylmercuric chloride per monomer in acetate buffer for 2 h. Protein was separated from unreacted reagent on the Sephadex column before it was assayed for free sulfhydryl groups in the usual manner.

**Crystallization**

Native and modified AAT's were crystallized by vapor diffusion against a solution of polyethylene glycol, PEG (McPherson, 1976; Arnone
et al., 1977). Each well in a glass spot plate contained 20 μL protein solution at a concentration of 30-50 mg/mL in 0.04 M sodium acetate, pH 5.4, and 20 μL 8% (w/v) PEG 6000 in the same buffer. The reservoir in a Petri dish contained 25 mL 8% PEG 6000 in the same buffer. The spot plate and reservoir were sealed in a plastic sandwich box with stopcock grease and set at 4° until crystals formed.

To crystallize mercury derivatives of the various sulfhydryl groups of AAT, 20 μL of a solution of native or modified AAT containing 120-200 nmol monomer was placed in a well with 20 μL 8% PEG 6000 containing 1.05 equivalents of p-chloromercuribenzoate or of methylmercuric chloride per sulfhydryl to be modified. Native AAT was set up with 1.05 or 2.1 equivalents of mercury per protein monomer in an attempt to prepare derivatives at Cys45 alone or at Cys45 and Cys82. AAT blocked at Cys45 with cyanide was used in an attempt to prepare a specific derivative of Cys82. AAT blocked at Cys45 and Cys82 with NEM, iodoacetamide, or cyanide was used in an attempt to prepare a derivative at Cys390. In all cases, the blocking groups were small and uncharged so that they would not disturb the structure of the protein nor interfere with intermolecular contacts in the crystal.

Reactions of Pyridoxal Phosphate Analogs with ApoAAT

ApoAAT (10\(^{-4}\)M monomer) was modified with pyridoxal 5'-sulfate (PLS) or with the 5'-trans-carboxyethenyl analog in 0.02 M triethanolamine buffer, pH 8.3. ApoAAT was allowed to react with one equivalent of coenzyme which was added from a 10 mM stock
solution. The reaction with PLS was allowed to proceed for 2 h at room temperature, the reaction with the carboxyethenyl analog for 20 h.

The concentration of the product of the reaction of PLS with apoAAT was calculated from the absorbance of the chromophore at low pH at 26 kK, using \( \varepsilon = 5.3 \times 10^3 \) (Yang et al., 1974).

**Enzymatic digestions**

The solution of modified apoAAT was passed over a column of Sephadex G25 (1.1 x 30 cm) equilibrated with 0.01 M HCl. The fractions containing the modified protein were easily identified by the intense fluorescence of the products at low pH.

The protein solution to be digested with chymotrypsin was adjusted to pH 7.3 with 0.5 M NaOH. Chymotrypsin, suspended in a small volume of water, was added in a 1/50 ratio by weight to the apoAAT. During the digestion the pH was maintained at 7.3 by a Radiometer TTT1 Autotitrator used as a pH-stat.

Digestion with pepsin was usually performed in 0.01 M HCl at pH 2.5. Pepsin was added from a freshly prepared solution of pepsin (10 mg/mL) in 0.02 M sodium acetate, pH 4.7, in a 1/100 ratio by weight to the apoAAT to be digested. After 1 h of digestion at room temperature, a second portion of pepsin was added in the same manner as the first, and digestion was allowed to proceed for 4 h longer. Digestion was terminated by the addition of a drop of pyridine, which raised the pH to above 5. The solution was then lyophilized.

Alternatively, the reaction mixture of apoAAT and coenzyme
analog in triethanolamine buffer was acidified by the addition of 1 M formic acid until the pH was lowered to 2.5. A solution of pepsin in .01 M formic acid was added in a 1/100 ratio by weight to the apoenzyme to be digested, and digestion was allowed to proceed at 37° overnight.

**Isolation of the modified peptides**

The lyophilized peptides from the pepsin digest of coenzyme-modified apoAAT were suspended in a small volume of 0.10 M acetic acid and applied to a column of Sephadex G25 (0.7 x 120 cm) equilibrated with 0.10 M acetic acid. An amount of digest representing up to 0.6 μmol AAT monomer could be applied at once and be separated adequately. Fractions of 0.8 to 1 mL were collected and the absorbances at 280 nm (35.8 kK) due to aromatic amino acids and at 386 nm (26.2 kK) due to the PLS chromophore were recorded.

Fractions that contained the PLS chromophore with a large number of other peptides were pooled, lyophilized, resuspended in about 1 mL of 0.10 M acetic acid, and applied to a column of Sephadex G15 (1.1 x 120 cm) equilibrated with 0.10 M acetic acid. Again, fractions of 0.8 to 1 mL were collected and analyzed for absorbance at 280 nm and at 382 nm.

In some preparations a single gel filtration step was used. The pepsin digest of up to 1.3 μmol of AAT was applied to a column of Sephadex G15 (2.2 x 120 cm). The peptides were eluted in 0.01 M formic acid. Fractions of 0.8 mL were collected and analyzed.

Further purification was achieved by electrophoresis on thin layers
of Sephadex G25 in formic acid/acetic acid/water (1:4:45, v/v), pH 1.9, on a Desaga/Brinkman double chamber electrophoresis apparatus. Thin layer plates were prepared according to instructions from Brinkman-Desaga. A thick slurry was prepared from 20 g Sephadex G25 superfine and 100 mL electrophoresis buffer. About 60 mL of the gel suspension was poured onto an extremely clean 40 x 20 cm glass plate, and spread to the edges with a glass rod. Two corners of the plate were lifted several cm above the bench and dropped to smooth minor irregularities in the gel layer. The resulting gel layer was smooth and shiny. It was left to air dry until it was quite dull but not cracked.

The lyophilized sample, usually containing about 0.4 μmole PLS chromophore, was suspended in 20 μL 0.10 M acetic acid and applied to the dried gel layer in a line about 2 cm from the anode end. Electrophoresis was run at 800 v, 35 ma for 5 h or longer, until the fastest-migrating fluorescent band approached the cathode end of the plate. Its progress was monitored periodically during the run with a UV lamp.

After electrophoresis was terminated, the fluorescent bands were scraped from the plate. The gel was suspended in a small volume of 0.1 M acetic acid and transferred to Pasteur pipets plugged with glass wool. The peptides were eluted from these columns with about 10 mL of 0.1 M acetic acid.

The final purification step was a second thin-layer electrophoresis at pH 4.7. The procedure was identical to that at pH 1.9, except that the gel buffer was 0.10 M ammonium acetate, pH 4.7. 1.0 M ammonium acetate buffer was used in the electrode chambers to
reduce the electrolytic heating there. The electrophoresis was run at 600 v, 25-40 ma, for about 5 h.

The peptides containing the carboxyethenyl chromophore were separated by gel filtration on the Sephadex G25 column and thin layer electrophoresis at pH 1.9 in the same manner as the PLS peptides.

At each step in the purification the samples were analyzed by high voltage paper electrophoresis at pH 1.9 and at pH 6.4. High voltage paper electrophoresis was done in the following way. For electrophoresis at pH 1.9, 5 µL of sample was applied in a narrow band 1 cm long along an origin line 15 cm from the anode end of a sheet of Whatmann 3MM paper, 46 x 57 cm. Without allowing the sample to dry, the paper was wetted with approximately 50 mL of pH 1.9 electrophoresis buffer, the same as that used for thin-layer electrophoresis, and the excess was blotted off. Electrophoresis was run at 2500 v for 45 min. Electrophoresis at pH 6.4 was performed in much the same way except that the origin line was in the center of the paper, the buffer was pyridine/acetic acid/water (3:100:897, v/v/v), and the electrophoresis was run at 1500 v for 75 min. After electrophoresis the papers were dried in a hood, the fluorescent spots were marked, and the papers were stained first with Cd** - ninhydrin stain (Yamada and Itano, 1966) and then with Ehrlich's stain for indole (tryptophan) (Zweig and Whitaker, 1969).

Characterization of the isolated peptides

Amino acid analysis of the purified peptides was performed on a Durrum D400 analyzer after hydrolysis of the peptides in 6 M HCl at
110° for 22 h. The amount of the PLS chromophore in each sample was determined from its absorbance at 382 nm at pH 3 using $\varepsilon = 5.3 \times 10^3$ before the sample was hydrolyzed.

The amino terminal residues were identified by dansylation and chromatography on polyamide sheets (Gray, 1972). Approximately 5 nmol of peptide in 10 μL 0.2 M NaHCO$_3$ was reacted with 10 μL dansyl chloride (2 mg/mL in acetone) for 1 h at 37°. The peptides were hydrolyzed in 50 μL 6N HCl in sealed tubes at 110° overnight. The hydrolyzed samples were dried in a vacuum desiccator over NaOH and P$_2$O$_5$, then suspended in 10 μL 50% pyridine. 5 μL of the sample was spotted on two polyamide sheets; reference dansylamino acids were added to one sheet. Chromatography was run first in H$_2$O/HCOOH (200:3, v/v), then in acetic acid/benzene (1:9, v/v) perpendicular to the first ascent. A third ascent, in the same direction as the second, was run in ethylacetate/acetic acid/ethanol/water (500:34:34:20, v/v). After the second ascent and after the third, the positions of the fluorescent spots were recorded in order to identify the dansylated amino acid from each peptide.

Digestion by aminopeptidase M, followed by high voltage paper electrophoresis or thin layer chromatography, was used to identify asparagine in an isolated peptide. A sample of peptide 4 containing 100 nmol of the PLS chromophore was digested with 3.5 μg aminopeptidase M in 0.15 M ammonium bicarbonate, pH 8, at 37° for 4.5 h. The digest was then subjected to high voltage paper electrophoresis at pH 1.9 and at pH 6.4 with serine, lysine, and asparagine standards.
A second sample of peptide 4, containing 25 nmol of PLS chromophore, was digested with 5 μg aminopeptidase M for 4 h at 37°. The digest was separated by thin layer chromatography on silica in chloroform/methanol/17% NH₄OH (2:2:1, v/v).
RESULTS

Crystallographic Studies of Aspartate Aminotransferase

The crystallography of cytosolic aspartate aminotransferase from pig hearts began when an exceptionally pure preparation of the enzyme crystallized on concentration. The purity of the enzyme is judged by the ratio of the protein absorbance at 280 nm to the PLP absorbance at 430 nm. A ratio of 9 is typical of most enzyme preparations, but the peak ratio of this preparation was 8.4. When the enzyme was being concentrated in 0.04 M sodium acetate, pH 5.4, by vacuum dialysis before being frozen for storage as usual, microcrystals formed. Because of the conical shape of the concentrator bag the protein became very concentrated in the tip where the crystals formed. The supernatant enzyme solution was taken to Arthur Arnone at the University of Iowa, and was set up to crystallize by the vapor diffusion technique of McPherson (1976). Large, well-formed crystals appeared within 3 to 5 days. Precession photographs of the diffraction pattern revealed that the crystals diffracted to at least 2.8 Å resolution.

Modification of sulfhydryl groups

The extent of modification of sulfhydryl groups of AAT by the various reagents are summarized in Table 1. Native AAT routinely showed 4.6 free sulfhydryls per monomer. Modification with iodoacetamide or NEM left 2.7 sulfhydryls per monomer, indicating that 1.9 sulfhydryls were modified. Excess DTNB modified 2.1 sulfhydryls
Table I. Determination of the extent of modification of the sulfhydryls of aspartate aminotransferase.

<table>
<thead>
<tr>
<th>Modification</th>
<th>free -SH</th>
<th>-SH modified</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>4.6±0.1</td>
<td></td>
</tr>
<tr>
<td>Iodoacetamide</td>
<td>2.7±0.1</td>
<td>1.9&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>1 equivalent of MeHgCl</td>
<td>1.7±0.1</td>
<td>1.0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>N-ethylmaleimide</td>
<td>2.7±0.2</td>
<td>1.9&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>MeHgCl (syncatalytic)</td>
<td>1.0±0.1</td>
<td>1.7&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>pMB (syncatalytic)</td>
<td>1.3±0.1</td>
<td>1.4&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.4 mM DTNB</td>
<td></td>
<td>2.1&lt;sup&gt;b&lt;/sup&gt;±0.1</td>
</tr>
<tr>
<td>1 equivalent of DTNB</td>
<td></td>
<td>1.0&lt;sup&gt;b&lt;/sup&gt;±0.1</td>
</tr>
</tbody>
</table>

<sup>a</sup>Determined by titration with DTNB after modification.

<sup>b</sup>Determined by TNB<sup>-</sup> release during the modification reaction.

per monomer of native AAT while 1 equivalent of DTNB per monomer reacted to completion.

Addition of 1.05 equivalents of methylmercuric chloride per monomer of iodoacetamide-blocked AAT resulted in the loss of 1.0 free sulfhydryl per monomer. On the other hand, syncatalytic modification of NEM-blocked enzyme with either methylmercuric chloride or pMB resulted in the loss of more than one free sulfhydryl per monomer.

**Heavy atom derivatives of aspartate aminotransferase**

The crystals of native aspartate aminotransferase that are used for crystallography are well-formed chisel-shaped plates (Arnone
et al., 1977). In order to give good data, the crystals must be at least 0.2 mm in the smallest dimension, but less than 1 mm, and have no flaws. Native AAT formed such crystals readily, but not the derivatives. Most of the crystals I obtained from modified proteins were either small plates, needles, or large clumps of twinned plates.

Native AAT crystallized with either 1 or 2 equivalents per monomer of methylmercuric chloride, and with a single equivalent of p-chloromercuribenzoate. No crystals formed from AAT with 1 or 2 equivalents of mercuric acetate, or with 2 equivalents of mercuribenzoate.

The cyanide derivative at cysteine-45 of AAT crystallized with no mercury reagent, and with 1 equivalent of methylmercuric chloride or mercuribenzoate, but did not crystallize with mercuric acetate. AAT modified at both cysteine-45 and cysteine-82 with cyanide crystallized with no mercury reagent and with methylmercury, but not with mercuric acetate or with mercuribenzoate.

Enzyme modified at the surface sulfhydryls with N-ethylmaleimide formed crystals with no mercury reagent, with methylmercuric chloride, and with mercuric acetate, but not with mercuribenzoate. A sample of NEM-modified AAT was modified syncatalytically with p-mercuribenzoate. Arnone and Rogers were not able to crystallize this derivative under the standard conditions, but did obtain small crystals in phosphate buffer at pH 7 by vapor diffusion. These crystals were not of the same crystal form as the native enzyme crystals, and so were not useful as a heavy atom derivative.

AAT modified at the surface sulfhydryls with iodoacetamide
crystallized with no mercury reagent and with one equivalent per monomer of methylmercuric chloride, p-chloromercuribenzoate, or mercuric acetate.

Small plates of native AAT grown in the presence of two equivalents of methylmercuric chloride, and of iodoacetamide-modified AAT grown with one equivalent of methylmercuric chloride showed intensity changes in their precession photographs, indicating that there was specific incorporation of mercury into the crystals. However, the crystals I obtained were too small to be used to collect data on the diffractometer.

One good crystal was obtained of NEM-blocked AAT grown in the presence of 1.05 equivalents of methylmercuric chloride. The crystal formed in two weeks after it was set up. The crystal was square, about 0.4 mm on a side, with a long needle attached to one side and a small flaw in one corner. Paul Rogers removed the needle and the flawed part of the crystal before mounting it for data collection. The crystal gave an interpretable Patterson map at 4.4 Å resolution (Figure 4) which showed there were one major and one minor mercury site in each monomer (Arnone and Rogers, personal communication).

**Crystallization of Apoaminotransferase**

A sample of aspartate aminotransferase was resolved by the usual method. The apoenzyme was passed over a column of Sephadex G25 (1.1 x 35 cm) in acetate buffer, and was concentrated to 21.2 mg/mL. The apoenzyme was set up to crystallize under the usual conditions
Figure 4  Harker section ($y = 0.5$) of the NEM and methylmercury derivative of aspartate aminotransferase at 4.4 Å resolution. The positions of the space group related vectors 1-1 and 2-2 are shown. The expected cross vector 1-2 appears as an intense peak on a y coordinate of 0.395. (Courtesy of Arthur Arnone and Paul H. Rogers, Department of Biochemistry, University of Iowa,)
with small crystals of native AAT present for seeds in some of the wells. The seeds were transferred from the well where they had formed on a dissecting probe. The apoenzyme crystallized on each seed to give well-formed crystals. The seeds could be seen as yellow areas near the center of the crystals; the apoenzyme on the edges was colorless. Several very small crystals consisting of only apoenzyme also formed in two of the wells with the seeded apoenzyme crystals. These crystals were used by Arnone and Rogers to seed additional set-ups of apoenzyme. A large well-formed crystal which grew from one of these apoenzyme seeds gave the diffraction data which was used in calculating a difference map of apoAAT versus native enzyme. A large negative peak was the outstanding feature of the difference map and clearly marked the position of the pyridoxal ring in the active site. The phosphate binding site in the crystal was not apparent, probably because a phosphate or sulfate ion was bound there (Martinez-Carrion, 1975; Verge et al., 1979).

Crystallization of other proteins

A few attempts were made to crystallize other proteins that were available, but none were successful. In all cases the sandwich box technique was used, with vapor diffusion against solutions of polyethylene glycol.

The \( \beta \) and \( \gamma \) subforms of AAT were set up to crystallize under the same conditions used for the \( \alpha \) subform. The \( \beta \) subform at 40 mg/mL formed only microcrystals. Later, Carol Metzler was able to grow a large crystal of \( \beta \)-AAT, which was determined to be isomorphous with
the α subform (Metzler et al., 1978). The γ subform did not crystallize when set up at 23.4 mg/mL, and eventually precipitated.

D-Serine dehydratase, a pyridoxal enzyme isolated from E. coli, was set up at 51.3 mg/mL in 0.1 M phosphate, pH 7.8, against a variety of PEG solutions. 4%, 8%, and 16% PEG 6000, 4%, 8%, and 12% PEG 4000, and 8% PEG 20,000 were all used. No crystals were formed under any of the conditions used, and the protein eventually precipitated.

Working with Louisa Tabatabai, I set up samples of phosphorylase kinase, 20 mg/mL in β-glycerol phosphate buffer, against 8% and 12% PEG 6000. The protein precipitated as soon as the PEG solution was added to each well.

Modification of Aspartate Aminotransferase with Coenzyme Analogs

**Reaction of pyridoxal sulfate with modified apoenzyme**

Aspartate aminotransferase was modified at cysteine-45 and cysteine-82 with N-ethylmaleimide as described in Methods. Titration of the modified enzyme with DTNB revealed 2.8 sulfhydryls per monomer, compared with 4.8 sulfhydryls per monomer in the unmodified enzyme. The NEM-modified enzyme was syncatalytically modified with DTNB and cyanide to block cysteine-390. Titration of this protein with DTNB revealed 2.1 sulfhydryls per monomer, indicating that approximately 70% of the enzyme molecules were modified at cysteine-390 with cyanide. A sample of AAT that was 50% modified with cyanide at cysteine-390 retained 80% of the enzymatic activity of the enzyme modified by NEM at the surface sulfhydryls only.
Pyridoxal phosphate could be resolved from the syncatalytically modified enzyme by the usual procedure except that longer incubation times were found to be necessary for more complete resolution. When the standard procedure was used, the ratio of the protein absorbance at 280 nm to the PMP absorbance at 330 nm was only 17, whereas a ratio of 40 or greater is acceptable (Furbish et al., 1969). When the incubation with 1 M phosphate was increased from 30 min to 3 h, the resulting apoenzyme had a ratio of 28. The spectrum of the modified apoenzyme (Figure 5) showed a small but unmistakable absorbance at 330 nm, indicating that the enzyme was still not completely resolved.

When a single equivalent of pyridoxal phosphate was added to the syncatalytically modified apoenzyme at pH 8.3, it was rapidly rebound to form a Schiff base absorbing at 363 nm, as with the unmodified apoenzyme. The absorbance at 363 nm reached a maximum within a few minutes and remained stable for at least 4 h.

Pyridoxal 5'-sulfate reacted with syncatalytically modified apoAAT in the same way as it reacted with unmodified apoenzyme. The spectrum of the product of the reaction, shown in Figure 5, has the same peak position and the same narrow shape with pronounced vibronic fine structure as the product of the reaction of PLS with unmodified apoenzyme (Yang et al., 1974).

Isolation of peptides modified by pyridoxal sulfate

Enzymatic digestions Yang et al. (1974) reported that the chromophoric product of the reaction of PLS with apoAAT is unstable when the protein was denatured by guanidine hydrochloride at high pH.
Figure 5 The reaction of $9.5 \times 10^{-5}$ M aspartate apoaminotransferase modified at cysteine-390 with cyanide with $9.2 \times 10^{-5}$ M pyridoxal sulfate. The numbers beside the curves indicate the time in minutes after mixing. The spectrum of the apoenzyme is also shown.
At pH 8.2 the absorbance at 402 nm disappeared overnight. The chromophore was much more stable at low pH (pH 5 or below) than at higher pH. The low pH form of the chromophore was found to be stable for many days in 6 M guanidine hydrochloride.

When the PLS-modified enzyme was denatured in 0.01 M HCl, then digested by trypsin at pH 7.3 for 5 h, the chromophore absorbance disappeared completely (Figure 6). The chromophore was also destroyed when digested by chymotrypsin or by a mixture of trypsin and chymotrypsin under the same conditions.

Attempts were made to isolate a modified peptide from chymotryptic digests on columns of Dowex 1-X2 eluted with pyridine-collidine-acetic acid buffers (Morino and Watanabe, 1969) and by paper chromatography and electrophoresis. Although there was no visible absorption spectrum that could be used to follow the PLS product, there was something in the digest with a blue-white fluorescence, which I supposed to be the PLS product. I attempted to follow this fluorescence through the separation procedure. These efforts were without success either in isolating a modified peptide or in identifying a PLS product.

The low pH form of the PLS chromophore proved to be stable under the conditions for digestion by pepsin. When the modified enzyme was digested with pepsin at pH 2.5 for 5 h, there was a slight shift of the absorbance maximum of the PLS chromophore from 386 to 382 nm (25.9 to 26.2 kK) and only 4% decrease in the peak height (Figure 6). With the addition of base, the solution became turbid, but the
Figure 6 Enzymatic digestion of pyridoxal sulfate-modified aspartate aminotransferase. a. 3.0 x 10^-5 M apoAAT modified by PLS, digested with chymotrypsin (1:100 w/w) for 5 h at pH 7.3. The pH was adjusted to 2.5 before the spectrum was recorded. b. 3.0 x 10^-5 M PLS-modified apoAAT digested with pepsin (1:100 w/w) for 5 h at pH 2.5.
The lyophilized peptides that resulted from the digestion of PLS-modified AAT by pepsin were first fractionated on a column of Sephadex G25 (0.7 x 120 cm). An amount of digest representing up to 0.6 μmol of AAT monomer could be applied at once to this column and be adequately separated. The peptides were eluted from the column in 0.1 M acetic acid. Fractions of 0.8 mL were collected and the absorbances at 280 nm, due to aromatic amino acids, and at 382 nm, due to the PLS chromophore, were recorded. A typical elution profile is presented in Figure 8. The PLS-peptides were rather small, since they emerged from the column after the bulk of the material. Recovery of the PLS chromophore from the Sephadex column was 76%.

The PLS chromophore was unaltered by gel filtration, as indicated by its spectrum (Figure 9). Furthermore, at pH 9, the chromophore changed to its form absorbing at 400 nm, with the characteristic narrow band.

High voltage paper electrophoresis at pH 1.9 of aliquots of the G25 fractions that contained the PLS chromophore showed that they were quite heterogeneous and contained several fluorescent species (Figure 10).

Fractions from the G25 column were pooled in three parts, representing the leading edge, the center, and the tail of the peak of chromophore content as it emerged from the column. Each pool was
Figure 7  Absorption spectrum of PLS-modified apoAAT after digestion with pepsin.  a. pH 2.5.  b. pH 8.1.
Figure 8  Fractionation of the peptic digest of 0.6 μmol PLS-AAT on a column of Sephadex G25 (0.7 x 120 cm) equilibrated with 0.1 M acetic acid.  a. Absorbance at 280 nm.  b. Absorbance at 382 nm, due to the PLS chromophore.
Figure 9 Absorption spectrum of a fraction containing the PLS chromophore after initial fractionation of PLS-AAT peptides on Sephadex G25. a. At pH 3.1. b. At pH 9.1.
Figure 10 High voltage paper electrophoresis at pH 1.9 of fractions from Sephadex G25 chromatography of the peptic digest of PLS-AAT. Each fraction (0.74 mL) was lyophilized and resuspended in 200 μL 0.1 M acetic acid. 5 μL aliquots were spotted for electrophoresis as described in Methods. (○ Ninhydrin spots. □□ Fluorescent spots.)
applied to a column of Sephadex G15 (1.1 x 120 cm) equilibrated with 0.10 M acetic acid. Again, fractions of 0.8 mL were collected and analyzed for absorbance at 280 and 382 nm. The elution patterns are shown in Figure 11.

Only the early PLS fractions from the G25 column were routinely subjected to the G15 fractionation because they were especially heterogeneous. Because of the tailing of the PLS peak on this column, only about 60% of the chromophobe that was applied was usually recovered.

After gel chromatography the peptides were purified by electrophoresis. Whatmann 3MM paper could not be used as the solid support for electrophoresis because it was not possible to recover material from the paper that showed the spectrum of the intact chromophore. Electrophoresis was run at pH 1.9, pH 4.7, or pH 6.4 with similar results: the chromophore could not be recovered. In one experiment, several samples of peptides that contained the chromophore were applied to a sheet of Whatmann 3MM paper that had been previously wetted with pH 1.9 electrophoresis buffer. Immediately after electrophoresis was stopped, the fluorescent spots were cut out and were eluted with 0.1 M acetic acid. Thus the samples were never allowed to dry or to contact dry paper. In another experiment, PLS peptides were spotted on paper and eluted without electrophoresis. In either case, the eluted material did not show the spectrum of the intact PLS chromophore.

The chromophore had been shown to be stable on Sephadex during gel chromatography, so a thin layer of Sephadex G25 superfine was
Figure 11 Elution patterns of peptide fractions from the Sephadex G25 column rechromatographed on a column of Sephadex G15 (1.1 x 120 cm) in 0.1 M acetic acid. Absorbance at 280 nm and at 382 nm are indicated by the numbers beside the curves.

A. Early fractions from the PLS chromophore peak on the G25 column.
B. Center of the PLS chromophore peak.
C. Trailing edge of the PLS peak.
used as the support for electrophoresis.

Further purification was achieved by electrophoresis on layers of Sephadex G25, superfine, in formic acid/acetic acid/water (1:4:45, v/v), pH 1.9, on a Desaga/Brinkman double chamber electrophoresis apparatus.

The sample was applied to the gel layer in a line about 2 cm from the anode end. Electrophoresis was run at 800 V, 35 mA, for 5 h or longer, until the fastest-migrating band with the fluorescence of the PLS chromophore approached the cathode end of the plate. Its progress was monitored periodically during the run with a UV lamp.

Material that eluted as a single peak from the Sephadex G25 column gave at least four bands with the white fluorescence of the PLS chromophore on electrophoresis at pH 1.9 (Figure 12). These bands were assigned numbers 1 through 4, in order of increasing mobility toward the cathode.

Immediately after electrophoresis was terminated, the areas of the gel that were fluorescent were scraped off the plate. The peptides were eluted from the gel samples with 10 to 15 mL 0.10 M acetic acid in Pasteur pipettes plugged with glass wool.

The material eluted from each of the fluorescent bands had an absorbance peak at 26 kK, so the chromophore could be at least partially recovered after thin layer electrophoresis.

Fractions 1 and 3 contained only small amounts of the chromophore, typically 2 to 3% of the amount that had been applied to the gel. These fractions were not obtained in sufficient quantity to permit further
Figure 12  A typical pH 1.9 electrophoresis plate under UV light. The origin line (ϕ), the anode (θ), and the cathode (θ) are indicated, along with the numbers assigned to the fluorescent bands.
purification, and paper electrophoresis showed that they each contained more than one peptide. Indeed, fraction 1 sometimes appeared to contain two fluorescent bands on the thin layer gel.

Fraction 4 contained 10 to 20% of the applied chromophore, while fraction 2 contained 20 to 30%. This was estimated from the absorbance at 26.2 kK of the material eluted from these bands after it had been concentrated to dryness on a rotary evaporator and taken up in a known volume of 0.1 M acetic acid. Samples that originated from the early PLS fractions from the Sephadex G25 column yielded more PLS chromophore in fraction 2 than fraction 4, while the center and tail of the G25 peak gave approximately equal amounts of chromophore in 2 and 4.

High voltage paper electrophoresis of a portion of fraction 4 at pH 1.9 gave a single intense fluorescent spot that stained faintly with ninhydrin, but was negative with Ehrlich's reagent for tryptophan. On electrophoresis at pH 6.4, two ninhydrin-positive spots appeared on the cathode side of the origin line, indicating that the PLS peptide was not completely pure. The more intense ninhydrin spot corresponded to the single fluorescent spot (Figure 13A and B).

On high voltage paper electrophoresis at pH 1.9, fraction 2 gave a single highly fluorescent spot that stained strongly with ninhydrin and that also reacted positively with Ehrlich's stain for tryptophan. Electrophoresis at pH 6.4 gave two spots, one highly fluorescent spot that had migrated a short distance toward the anode and that reacted strongly with both ninhydrin and Ehrlich's stain, and a faintly
Figure 13  High voltage paper electrophoresis of samples of fraction 2 and fraction 4 from thin-layer electrophoresis at pH 1.9. (♀) Fluorescent spots. (○) Ninhydrin spots. (♀) Tryptophan.

A. Electrophoresis at pH 1.9. B. Electrophoresis at pH 6.4.
fluorescent spot on the cathode side that gave a faint ninhydrin spot (Figure 13A and B).

The spectrum of fraction 2 is shown in Figure 14. The absorbance at 382 nm of the PLS chromophore was present at low pH. In addition, there was a prominent peak at 280 nm that had the position and shape characteristic of tryptophan (Metzler et al., 1972). When the concentration of tryptophan was calculated from the absorbance at 35.8 kK using $\varepsilon = 5.57 \times 10^3$ (Metzler et al., 1972), it was found that tryptophan was present in different preparations at four to five times the concentration of the PLS chromophore.

The tryptophan-containing peptides and the PLS product were separated by a second thin layer electrophoresis in 0.1 M ammonium acetate at pH 4.7. The PLS chromophore was present in the most rapidly migrating fluorescent band. Tryptophan was in a slower-moving band that could be seen as a faintly yellow fluorescent band about half-way between the PLS and the origin. The yield of the chromophore was about 48% of the amount that was applied.

On high voltage paper electrophoresis at pH 1.9, the purified peptide 2 again gave a single fluorescent spot that was ninhydrin-positive, but was negative with Ehrlich's stain. Electrophoresis at pH 6.4 gave only one spot on the cathode side of the origin which was fluorescent and stained faintly with ninhydrin. The spot seen previously on the anode side was no longer present.
Figure 14  Spectrum of fraction 2 from thin layer electrophoresis at pH 1.9. a. pH 3.  b. pH 10.
Characterization of the isolated peptides

Peptides isolated by electrophoresis  Samples of peptide 2 and peptide 4 were isolated by a combination of gel chromatography and thin-layer electrophoresis, as described above.

Spectra of the chromophore  The spectrum of peptide 4 is shown in Figure 15. At pH 3.0, the spectrum showed a peak at 382 nm, with a narrow spike at 281 nm. When the pH was raised to 10.4 by the addition of NH₄OH, the chromophore peak shifted to 400 nm, as it does in the intact protein. The spike at 281 nm was no longer present, as the absorbance generally increased below 350 nm. At pH 10.4, the chromophore peak diminished in a first-order process with $t_{1/2}=20$ min (Figure 16). Accompanying the decrease at 400 nm were an increase around 310 nm and a small decrease around 280 nm. The spectrum of the final product had no identifiable peaks in the region of 500 to 250 nm.

The spectrum of peptide 2 is shown in Figure 17. The PLS chromophore peak at 382 nm was present, as in Figure 14, but the tryptophan spectrum was not. This was expected, since the purified peptide did not react with Ehrlich's stain (see Figure 13). The PLS peak shifted to 400 nm when the pH was raised to 10, as it did in the intact protein and in peptide 4. The chromophore of peptide 2 was unstable at pH 10, and its spectrum decayed (Figure 18) in the same manner as the chromophore of peptide 4. Again, the spectrum of the final product had no identifiable peaks in the region of 500 to 250 nm. The rising absorbance in the ultraviolet region of the
Figure 15  Spectrum of fraction 4 from thin-layer electrophoresis at pH 1.9.  a. pH 3.  b. pH 10.4.
Figure 16 Decay of the PLS chromophore of peptide 4 at pH 10. The numbers beside the curves indicate the time in minutes after the addition of NH₄OH to raise the pH from 3 to 10.
Figure 17 The spectrum of peptide 2 after electrophoresis at pH 4.7.
Figure 18  Decay of the PLS chromophore of peptide 2 at pH 10. The numbers beside the curves indicate the time in minutes after the addition of NH₄OH to raise the pH from 3 to 10.
spectrum is probably due to contamination. The peak at 240 nm does not correspond to any aromatic amino acid.

A sample of fraction 2 from thin layer electrophoresis at pH 1.9 was incubated at pH 10 until the PLS chromophore absorbance at 400 nm was destroyed. On high voltage paper electrophoresis at pH 1.9, the treated peptide had the same migration as the untreated PLS peptide and the tryptophan peptide. At pH 6.4, the treated PLS peptide migrated with the untreated peptide (Figure 19). Although the PLS chromophore is altered by base treatment, it apparently remains attached to the peptide.

**Amino acid analysis** The results of the amino acid analysis of samples of peptide 2 and peptide 4 that were isolated by gel chromatography and electrophoresis are given in Table 2. The amount of the PLS chromophore in each sample was calculated from the absorbance at 382 nm, using $c = 5.3 \times 10^3$ (Yang et al., 1974).

Peptide 2 contained aspartate, serine, glycine, leucine, phenylalanine, and lysine in amounts approximately equal to the amount of PLS chromophore. Glutamate, alanine, valine, and isoleucine were also present as significant contaminants but at less than half the amount of PLS.

Peptide 4 contained only 3 amino acids: aspartate, serine, and lysine. The yields of these amino acids were almost equivalent to the calculated content of PLS chromophore. The most significant contaminants were glycine, valine, tyrosine, and arginine, but the yields of these amino acids were less than half the amount of PLS.
Figure 19  Fraction 2 from thin-layer electrophoresis at pH 1.9.  
1: untreated.  2: treated with base to destroy the PLS chromophore.  
A. Electrophoresis at pH 1.9.  B. Electrophoresis at pH 6.4.
Table 2. Amino acid analysis of peptide 2 and peptide 4 isolated by gel chromatography and thin layer electrophoresis.

<table>
<thead>
<tr>
<th></th>
<th>Peptide 2</th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>nmol relative to PLS</td>
<td>nmol relative to PLS</td>
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<tr>
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<td>20</td>
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</tr>
</tbody>
</table>

a Not detected in the absorbance spectrum at 280 nm.

b Calculated from the absorbance at 382 nm at pH 3, using \( \varepsilon = 5.3 \times 10^3 \) (Yang et al., 1974).
Amino terminal analysis

Amino terminal analysis was performed on samples from the same peptide preparations that were used for amino acid analysis in Table 2. The results indicated that serine was the amino terminal residue in each peptide, but that peptide 4 was not completely pure. N-Dansylserine was the most intense spot in each chromatogram. Each chromatogram also contained two intense spots that were identified as dansyl-NH₂ and dansyl-OH. The chromatogram of peptide 4 contained two additional spots which, from their positions and colors, were identified as N-dansylvaline and O-dansyl-tyrosine. Both valine and tyrosine were indicated in the amino acid analysis (Table 2) as significant contaminants.

Alternative isolation procedure

Robert D. Scott later found that the PLS peptides could be isolated on a large scale in good yield by chromatography on Sepharose P2, TLC on silica gel in butanol/acetic acid/water (4:1:1 v/v) and thin-layer electrophoresis at pH 1.9. We isolated peptides 2, 3, and 4 in this manner from 4.5 μmol PLS-modified AAT. I analyzed these peptides with the following results.

Amino acid analysis

The amino acid analysis of peptides 2, 3, and 4 are presented in Table 3. The amino acid contents of peptide 2 and of peptide 4 are in agreement with those in Table 2. Each peptide contains aspartate, serine, and lysine. In addition, peptide 3 contains phenylalanine, and peptide 2 has glycine, phenylalanine, and leucine. The amounts of the contaminating amino acids are lower than in Table 2, and are less than 20% of the PLS content in
Table 3. Amino acid analysis of peptides 2, 3, and 4 isolated by gel chromatography, TLC, and TLE.

<table>
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<th>amino acid</th>
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<tr>
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<td>cys</td>
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<tr>
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</tbody>
</table>

^aFrom absorbance at 280 nm (35.8 kK) See Figure 20.

^bDetermined from absorbance at 382 nm (26.2 kK), using c=5.3 x 10^3 M^-1 cm^-1 (Yang et al., 1974). See Figure 20.
all cases except histidine. The amino acids for which no value is
given were all less than 10% of the PLS content.

**Spectra of the isolated peptides** The spectrum of each
of the isolated peptides is shown in Figure 20. These spectra all
have the same appearance as the spectrum in Figure 15. The spikes
at 281 nm are all lower relative to the 382 nm peak than the spike
in Figure 15, as there is generally less absorbance in the ultraviolet
region.

**N-terminal analysis** Samples of peptides 2, 3, and 4 were
analyzed for the amino terminal residue by dansylation and thin-layer
chromatography. Peptide 2 (Figure 21A) gave N-dansylserine as the
major amino acid product, plus a small amount of N-dansylaspartate.
The aspartate content of this peptide was rather high, so this may
reflect a small contaminant. Peptide 3 (Figure 21B) showed N-dansyl-
phenylalanine and N\(^-\)dansyllysine. Peptide 4 (Figure 21C) gave N-
dansylserine as the major amino acid product, plus a negligible amount
of N-dansylglycine. An error was made during the chromatography of
the samples from peptide 2 and peptide 3, so that their chromatograms
are different from that of peptide 4. However, standards were run in
parallel with each sample, so that the results are interpretable.
All the samples showed dansyl-NH\(_2\) and dansyl-OH, which are the reaction
products of dansyl chloride with ammonia and water, respectively. In
addition, each sample had an orange spot that migrated with the sol-
vent front on the third ascent.
Figure 20  Spectra of PLS peptides isolated by gel chromatography, thin-layer chromatography, and thin-layer electrophoresis, in 0.01 M formic acid, pH 2.  A. Peptide 2.  B. Peptide 3.  C. Peptide 4.
Figure 21A Thin-layer chromatography of dansyl amino acids from PLS peptides. (Yellow fluorescence. Blue fluorescence. Orange fluorescence.) Peptide 2.
Figure 21B  Peptide 3.
Figure 21C  Peptide 4.
Asparagine  Two separate samples of peptide 4 were digested with aminopeptidase M. This peptidase cleaves proteins and peptides to free amino acids without converting amide residues to carboxylic acids, as acid hydrolysis does. After digestion one sample was subjected to high voltage paper electrophoresis at pH 1.9 and at pH 6.4 along with standards of asparagine, lysine, and serine. The results are shown in Figure 22. The two major ninhydrin spots are serine and asparagine, but the lysine spot was much less intense than these two. There were three additional spots, one of which corresponded to a faint fluorescence. These spots may have been the products of incomplete digestion, or contaminants. The fluorescent and ninhydrin spot may have been a single modified lysine in very low yield.

The second sample of peptide 4 that had been digested with aminopeptidase M was analyzed by thin-layer chromatography (Figure 23). The three ninhydrin spots corresponded to lysine, asparagine, and serine. Thus peptide 4, and presumably also peptide 2 and peptide 3, contains asparagine rather than aspartic acid.

Histidine  Because the amino acid analyses of several samples of PLS peptides indicated that histidine was present as a significant contaminant, the histidine content of a sample of peptide 3 was determined independently by reaction with Pauly's reagent for imidazole (Ray, 1967). The amino acid analysis of this peptide (Table 3) included 0.3 mol histidine per mol PLS. A 62-nmol sample of peptide, which should have contained 19 nmol histidine, in 2 mL of water was reacted with 0.5 mL of 0.4% diazotized sulfanilic acid
Figure 23  Thin-layer chromatography of peptide 4 after digestion by aminopeptidase M. Chromatography was run on a silica gel plate in chloroform/methanol/17% ammonia (2:2:1, v/v). 4: Digest of peptide 4. Std: standard of serine, lysine, aspartic acid, and asparagine. (Ninhydrin spots.)
in 10.8% sodium carbonate. The resulting absorbance at 510 nm was compared to a standard curve prepared from solutions of histidine. The absorbance at 510 nm of the peptide sample corresponded to a total of 5 nmol of histidine. This is much less than the amino acid analysis indicated. The reaction of diazotized sulfanilic acid with histidine standards was not instantaneous. The absorbances reached a maximum value in about 2 min. However, the absorbance of the peptide sample did not change in this time. Since Pauly's reagent reacts with phenol as well as imidazole, the small absorbance may have been due to the reaction of the reagent with the phenolic hydroxyl of PLS and the sample may contain no histidine at all.

Attempts to obtain a smaller modified peptide After digestion of peptide 4 with aminopeptidase M for 4 h at pH 8, the spectrum of the PLS chromophore was no longer observed. This was expected, since the chromophore was known to be unstable at this pH. On the other hand, incubation of a mixture of PLS peptides with aminopeptidase M at pH 5.9 overnight did not alter the electrophoretic mobilities of the peptides. Therefore, it seems that the peptidase is not active in the pH range where the PLS chromophore is stable, and that it is not possible to obtain a smaller modified fragment by this means.

In the same manner, digestion of a mixture of PLS peptides with pepsin at 37° overnight did not change the electrophoresis pattern of the sample. It had been hoped that this treatment might convert all the PLS peptides to the smallest peptide, peptide 4.
Peptides modified by the carboxyethenyl analog

ApoAAT was modified by the 5'-trans-carboxyethenyl analog of PLP (Miura and Metzler, 1975). The modified enzyme was digested with pepsin under the same conditions used for the digestion of the enzyme modified by PLS. The absorption of the low-pH form of the chromophore at 395 nm (25.3 kK) was almost unchanged upon digestion by pepsin. At pH 9, the chromophore converted to its form absorbing at 412 nm (24.3 kK). These spectra are shown in Figure 24.

The whole digest was fractionated on a column of Sephadex G25 (0.7 x 120 cm). The elution pattern is shown in Figure 25. Comparison of Figure 25 and Figure 8 reveals great similarity. Not only are the absorbances at 280 nm similar, but the chromophore-containing peptides emerge from the column at the same position.

The fractions that contained the chromophore of the analog were pooled, concentrated, and subjected to thin layer electrophoresis at pH 1.9, along with a similar sample of PLS-containing peptides. The yellow fluorescent peptides of the carboxyethenyl analog sample migrated approximately the same distances as the white fluorescent PLS peptides. The number and relative intensities of the fluorescent bands were the same: bands 2 and 4 were the major bands, and bands 1 and 3 were less intense. The peptides containing the carboxyethenyl analog were not recovered from this step in sufficient quantity to permit further characterization.
Figure 24  Spectrum of apoAAT modified by the carboxyethyl analog after digestion by pepsin.  a. pH 2.4.  b. pH 9.
Figure 25 Fractionation of the peptic digest of apoAAT modified by the carboxyethenyl analog on a column of Sephadex G25 (0.7 x 120 cm) in 0.1 M acetic acid. Absorbance at 280 nm and at 395 nm (25.3 kK) are indicated by the numbers beside the curves. The arrow marks the peak of PLS peptides on the same column.
DISCUSSION

Crystallographic Studies of Aspartate Aminotransferase

The crystallization of aspartate aminotransferase in a form suitable for X-ray crystallography has opened a large and important field in the study of this enzyme. With the structure of the enzyme almost in hand, it will soon be possible to learn many new details about its means of catalysis. Because it is possible to obtain isomorphous crystals of a number of enzyme-substrate and enzyme-inhibitor complexes (Metzler et al., 1978), it will be possible to detect changes that occur in the structure of the enzyme between stages in the catalytic cycle. There is a large body of evidence that suggest that such changes occur -- syncatalytic modification of cysteine-390 (Birchmeier et al., 1973a), changes in the number of exchangeable protons (Pfister et al., 1978), polarized light absorption spectra of crystalline enzyme-substrate complexes (Metzler et al., 1979) -- but soon we will be able to see what the changes are.

The several isozymes of aspartate aminotransferase are the only pyridoxal phosphate enzymes whose structures are known, other than phosphorylase where the function of the coenzyme is not known. Therefore these enzymes will be a model for the interactions of the protein, the coenzyme, and the substrate during catalysis. The crystallographic studies may also provide insights into the methods of catalysis by enzymes in general.
Crystalline heavy atom derivatives

The most successful method of preparing crystalline heavy atom derivatives of proteins has been to soak crystals of the native protein in solutions of the heavy atom reagent. The heavy atom reagent diffuses through solvent channels in the protein crystal to suitable binding sites. The resulting crystals are likely to be isomorphous with the native crystals, but the heavy atom sites are often non-specific and disordered. An alternative technique, which I used, is to react the protein in solution with a heavy atom reagent which forms covalent bonds with the protein, and then to crystallize the modified protein. This technique is often unsuccessful because the heavy atom reagent changes the structure of the protein or alters interactions between protein molecules so that the modified protein will not crystallize or form crystals that are not isomorphous with crystals of the native protein. However, with the methylmercury derivative of aspartate aminotransferase, this method works well.

Native AAT, AAT modified at cysteine-45, and AAT modified at cysteine-45 and cysteine-82 all crystallized in the presence of one or more of the mercury reagents that were tried. Methylmercury derivatives were the most readily crystallized, but mercuribenzoate and mercuric acetate derivatives crystallized in some cases as well. Most of the crystals that formed for me were too small or too irregular to be used for crystallography, but the fact that they formed at all is encouraging. Using small crystals of native AAT as seeds, it should be possible to grow larger crystals of these heavy atom derivatives.
The crystal of NEM-modified AAT that was grown in the presence of one equivalent of methylmercuric chloride per protein monomer was the first good heavy atom derivative crystal of AAT. This crystal gave an interpretable Patterson map, indicating that there were a limited number of heavy atom sites in the unit cell. The peaks in the section of the Patterson map of this crystal, shown in Figure 4, are from the two major sites in each dimer. The two minor sites had a much lower occupancy, and so did not show up clearly.

The Patterson function represents a map of interatomic vectors. A peak in the Patterson map has a weight proportional to the number of electrons in the two atoms represented by that peak. This means that vectors between pairs of heavy atoms give strong peaks in the Patterson map. However, since N heavy atoms in a unit cell give rise to $N^2$ peaks in the Patterson map, it is important to have only a few specific heavy atom sites (Blundell and Johnson, 1976).

A Harker section (Figures 4 and 26) is a section of the Patterson map where vectors between symmetry-related heavy atoms appear. For the space group $P2_12_12_1$ there are three Harker sections, at $x=0.5$, $y=0.5$, and $z=0.5$. The Harker section $y=0.5$, calculated at 3.5 Å resolution for a crystal of native AAT grown in the presence of two equivalents of methylmercuric chloride per monomer, is shown in Figure 26. There are four peaks of differing intensities in this section, indicating that there are two major and two minor mercury sites on each dimer. A diagram of the relative positions of these four sites is shown in Figure 27. The distances from sites A to B and
Figure 26  Harker section (y=0.5) of a crystal of native aspartate aminotransferase containing two equivalents per monomer of methylmercuric chloride. (Courtesy of Arthur Arnone and Paul H. Rogers, Department of Biochemistry, University of Iowa.)
Figure 27 Calculated distances between mercury sites in a crystal of aspartate aminotransferase containing two equivalents of methylmercuric chloride per protein monomer. (Courtesy of Arthur Arnone and Paul H. Rogers, Department of Biochemistry, University of Iowa.)
from A' to B', and from A to B' and A' to B are in agreement within experimental error. This shows that there are two pairs of equivalent mercury sites in the dimer related by a two-fold axis of symmetry (A. Arnone and P.H. Rogers, personal communication).

It had been supposed that mercury reagents, such as methylmercuric chloride and p-chloromercuribenzoate, would react with the sulfhydryl groups of AAT in the same order as other sulfhydryl reagents: cysteine-45 first, then cysteine-82, cysteine-390 under syncatalytic conditions or at low pH, cysteine-191 and cysteine-252 only under denaturing conditions (Wilson et al., 1974; Zufarova et al., 1973). However, Rogers and Arnone found that the same major and minor sites were occupied by methylmercury whether or not cysteine-45 and cysteine-82 were previously blocked by NEM. As the polypeptide chain was traced the major methylmercury site was identified as cysteine-191 and the minor site as cysteine-82. NEM was supposed to have blocked cysteine-82, as well as cysteine-45, but less than two sulfhydryls per monomer were lost during the incubation with this reagent (Table 1). A fraction of these residues must have remained unmodified and available to methylmercury. The occupancy of this site in the crystals of NEM-modified AAT was very low.

P.H. Rogers and A. Arnone have grown well-formed crystals of AAT in the presence of more than two equivalents of methylmercuric chloride per monomer, but the crystals were not stable and cracked when they were warmed to room temperature. It is therefore not possible at this point to say whether methylmercury can be forced
onto additional sites or not. Rogers and Arnone have also obtained good crystals of AAT modified with p-chloromercuribenzoate. There are two sites for this reagent. The major site has been identified as cysteine-82, which is the minor site for methylmercury, and the minor site is cysteine-45. Although syncatalytic modification of cysteine-390 remains an attractive possibility, it has not yet given any suitable crystals.

**Locations of reactive residues**

An electron density map at 2.7 Å resolution has been calculated using the methylmercury and p-mercuribenzoate derivatives. Most of the polypeptide chain has been traced on this map, partially by comparing it to the published structure of mitochondrial AAT (Eichele et al., 1979a).

From the map at 2.7 Å resolution, the methylmercury and p-mercuribenzoate sites listed above were identified. Cysteine-191, the major methylmercury site, is located on a bend at the end of a β-strand, buried within the protein. Its location in such a rigid structure explains why this residue was not accessible to most sulfhydryl reagents except in the denatured protein (Wilson et al., 1974; Polyanovsky et al., 1973a; Zufarova et al., 1973). The small size of methylmercury and the nonpolar nature of its methyl group must enable this reagent to penetrate into the hydrophobic core of the protein.

Methylmercury and p-mercuribenzoate both bind to cysteine-82. This residue is located in a pocket on the surface of the protein.
Methylmercury binds between cysteine-82 and threonine-81, while mercuribenzoate binds in the midst of cysteine-82, arginine-85, and leucine-310. The leucine and arginine residues may be interacting with the aromatic ring and carboxyl group of the reagent, or mercuribenzoate may bind in this location in order to be oriented toward the solvent where there is room for its large benzoic acid moiety.

The minor mercuribenzoate site, cysteine-45, is also located on the surface of the protein. The mercury position is in the solvent with threonine-42 and possibly tryptophan-48 nearby.

Many researchers have drawn inferences about the structure of AAT from indirect evidence such as the reactivity of a residue, or the behavior of a paramagnetic or fluorescent group attached to a residue. In general, these predictions have been quite accurate. The locations of the five cysteine residues of AAT in the electron density map confirm the earlier proposals. Cysteine-45 and cysteine-82 are located on the surface of the protein, cysteine-390 is located near the active site, cysteine-191 and cysteine-252 are located on the β wall, buried in the core of the protein (Zufarova et al., 1973; Polyanovsky et al., 1973a; Wilson et al., 1974). Polyanovsky et al. (1973b) and Polyanovsky and Misharin (1974) calculated that cysteine-45 and cysteine-82 were about 2.0 to 2.3 nm apart and that each was about 2.5 nm from the active site. In the electron density map, cysteine-45 is located more than 3 nm from cysteine-82, but is only about 2.7 nm from cysteine-82 of the other subunit of the dimer. Cysteine-45 and cysteine-82 are about 1.7 and 2.1 nm, respectively, from the
Deev et al. (1978) concluded that cysteine-390 and tyrosine-70 must be about 0.5 nm from the ε-amino group of lysine-258, since difluorodinitrobenzene crosslinks lysine-258 with either of the other two residues in apoAAT. Tyrosine-70 of the other subunit of the dimer is 0.5 nm from lysine-258, but cysteine-390 is about 1.0 nm away. Boettcher and Martinez-Carrion (1978) calculated that a paramagnetic probe attached to cysteine-390 was about 0.8 to 0.9 nm from the coenzyme. This is reasonable in view of the location of cysteine-390 in relation to the active site.

Two arginine residues, residue 386 of the subunit that forms the major part of the active site and residue 292 of the other subunit of the dimer, are located at either end of the binding site for α-methylaspartate. However, from the 3.0 Å map it is not possible to tell which binds the ω-carboxyl of the substrate, as proposed by Gilbert and O'Leary (1975). Khomutov et al. (1969) found evidence of a threonine residue near the phosphate of the coenzyme. In the electron density map threonine-109 is located close to the phosphate and probably forms a hydrogen bond to it.

Ivanov and Karpeisky (1969) concluded from studies of the circular dichroism of the enzyme that a tyrosine residue formed a hydrogen bond to the ring nitrogen of PLP. The electron density map denies this possibility: The ring nitrogen is bonded to aspartate-222. Tryptophan-140 is located near the coenzyme ring and may be the residue that is perturbed on binding PLP to give rise to
the changes in the circular dichroism (Yang et al., 1975). On the other hand, since binding of PLP to one subunit of the apo-enzyme dimer causes a conformational change in the other subunit (Schlegel and Christen, 1974; Arrio-Dupont and Cournil, 1975), the perturbed residue may be tryptophan-295 which is located at the subunit interface, close to the molecular diad.

The observed failure of apoenzyme modified by tetranitromethane to recombine with PLP (Turano et al., 1971) may have been due to nitration of tyrosine-225, which binds the phenolic oxygen of the coenzyme, or of tyrosine-70, which forms a hydrogen bond to the phosphate group.

The identity and location of the reactive histidine residue are not clear from the electron density map. Histidine residues 143, 189, and 193 are located closer to the active site than the others, but are deeply buried within the protein and so would not be accessible to photosensitizing dyes or chemical reagents. Histidines 317, 352, and 378 are all located on the surface of the protein, but are far from the active site. Histidine-68 is both inaccessible and too far from the active site. Morino et al. (1979) have concluded that the critical histidine is residue 405, but this residue is located in the α-helix which forms the C-terminal segment and is far from the active site. What is clear is that there is no histidine located in the active site to remove the α-proton of the amino acid substrate, contrary to some suggestions (Peterson and Martinez-Carrion, 1970).
Modification by Coenzyme Analogs

In earlier work by Yang et al. (1974) it was proposed that pyridoxal sulfate reacts at the active site of the apoenzyme of aspartate aminotransferase since the enzyme is inactivated in the course of the reaction and can no longer bind its natural coenzyme, pyridoxal phosphate. The absorption spectrum taken within a few minutes of mixing the coenzyme analog with the apoenzyme suggests that a Schiff base is formed initially, but later spectra suggest a more complicated reaction follows. The purpose of this research is to investigate this reaction further to identify the amino acid residue(s) of the protein that are involved in the reaction, and to gain information about the structure of the reaction product.

Since PLS appears to react at the active site of apoAAT, it was presumed that PLS reacted first with lysine-258 to form a Schiff base. Cysteine-390 was thought to be the residue most likely to react with the Schiff base because it was known to be located near the active site and because the reaction of PLS with cysteine in solution showed spectral characteristics remarkably similar to those of the enzymatic reaction. If cysteine-390 were involved in the reaction, then modification of this residue would be expected to interfere with the reaction. In the clearest case, PLS would be able to form a Schiff base with the modified apoenzyme but not be able to react further.

ApoAAT 70% modified at cysteine-390 with cyanide reacted with PLS to form a species absorbing at 402 nm (24.9 kK) at pH 8.3. The
absorbance band was narrow with pronounced vibronic fine structure and closely resembled the absorbance of the product of the reaction with the unmodified apoenzyme. The 402-nm peak formed faster and reached a higher absorbance in the reaction with the modified apoenzyme than in the reaction with the unmodified apoenzyme. Yang et al. reported that the 402-nm peak reached a maximum molar absorptivity of $1.0 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$ in about 2 h. With the modified apoenzyme (Figure 5), this peak reached a maximum molar absorptivity of $1.4 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$ in only 1 h, and the initial complex spectrum was not recorded. However, another sample of apoAAT, which was only 50% modified at cysteine-390, reacted more slowly with PLS than the sample in Figure 5, and the spectral changes more closely resembled those described for the reaction with the native apoenzyme. In either case, it is apparent that PLS and apoAAT that has been modified at cysteine-390 react in a way not fundamentally different from the reaction of the unmodified apoenzyme. It is unlikely that the reaction proceeded after the elimination of the blocking group to regenerate the sulfhydryl because the reactions that modified the sulfhydryl result in its oxidation. Cyanide could not be eliminated without leaving the sulfur atom severely electron-deficient. The thiocyanide itself is not a nucleophile and so could not mimic the sulfhydryl in the reaction. Furthermore, the 2.7 Å electron density map of native AAT indicates that cysteine-390 is located 10 Å away from the active site, too far to participate in the reaction. Thus, cysteine-390 is not the unknown nucleophile that reacts with
Isolation of modified peptides

The specificity of bond cleavage by trypsin or chymotrypsin make these enzymes the first choices for hydrolysis of a modified protein in locating sites of modification. Trypsin would be expected to cleave a peptide of twenty-five residues from the active site of AAT, while chymotrypsin would most likely leave a tetrapeptide. Since a tetrapeptide would be easily characterized and identified within the amino acid sequence, chymotrypsin was preferred for this study. Unfortunately the PLS chromophore proved to be unstable under the conditions for digestion by either chymotrypsin or trypsin. During the digestion the visible absorption spectrum of the PLS chromophore was lost. The chromophore presumably converted to a form whose absorption is obscured by the 280-nm band of the aromatic amino acids, as it does when the protein is denatured at high pH. Attempts to isolate a peptide containing this new form of PLS were unsuccessful. Because its absorption spectrum was not characterized, there was no means of quantifying the desired material or even of locating it definitely. Yang et al. (1974) speculated that when the 400 nm band disappeared after the modified enzyme was denatured in 6 M guanidine hydrochloride at high pH, a reaction occurred that led to a shift of the absorption band of the chromophore into that of the aromatic amino acids of the protein. Such an absorption spectrum, even if it were well-characterized, would be of little use in following the chromophore through an isolation procedure because of the inter-
ference of the absorptions of tryptophan and tyrosine. The presence of pyridine and collidine in the buffers used to elute peptides from the Dowex-1 column made the ultraviolet region of the absorption spectrum completely inaccessible.

There was a species with a blue-white fluorescence present in the chymotrypsin digest of PLS-modified apoAAT. I attempted to isolate this species, thinking that it may be a peptide modified with the altered PLS chromophore. However, peptide maps of fractions from the Dowex-1 column that contained the blue-white fluorescence showed that the fluorescence was not associated with any ninhydrin-positive material, and so this course was abandoned.

As reported by Yang et al. (1974), the product of the reaction of PLS with apoAAT is stable at low pH for many days, even after the protein is denatured in 6 M guanidine hydrochloride. Therefore pepsin, which is active at low pH, was chosen to digest the modified protein, although it lacks the specificity of trypsin or chymotrypsin.

The PLS chromophore survived digestion by pepsin at pH 2.5 for 5 h with almost no decrease in the intensity of its absorbance. The absorption peak shifted slightly during the digestion, from 386 to 382 nm (25.9 to 26.2 kK), but this was probably due to the disruption of the protein structure and loss of its interactions with the chromophore rather than any destruction of the chromophore. The environment of the coenzyme in the active site of the enzyme is thought to be rather hydrophobic. The structure of the coenzyme pocket is destroyed as the protein is digested, leaving the PLS
chromophore in an aqueous environment. After digestion, the chromophore could be converted to its form absorbing at 400 nm by adjusting the pH to 8. This indicates that the PLS chromophore is unaltered by treatment by pepsin.

Two peptides that contained the PLS chromophore were isolated from the pepsin digest using gel chromatography and thin layer electrophoresis. Gel chromatography on Sephadex G-25 or G-15 separated the modified peptides from most of the other peptides in good yields. The losses could be explained by a very low absorbance at 382 nm in all the fractions and by the tailing fractions which were discarded. Electrophoresis at pH 1.9 separated the modified peptides into at least four bands. Peptide 4 was occasionally obtained in an almost pure state from this step. A second electrophoresis at pH 4.7 was used to further purify peptide 2 and peptide 4. The fluorescent bands on the pH 4.7 electrophoresis plates were often very broad. Because of this, the contaminating peptides were often not well-separated from the desired peptide, and the yields were poor. The losses on electrophoresis were in part due to the spreading of the bands, since only the center of a band was taken to avoid including contaminating peptides. In order to keep the sample application line narrow, the samples were applied in very small volumes. This led to significant losses in the transfer, usually amounting to about 10% of the sample.

The alternative method for the isolation of the modified peptides, using gel chromatography, thin layer chromatography,
and thin layer electrophoresis, was used for a large scale preparation of peptides. Peptides 2 and 4 that were isolated in this manner were the same as those isolated by the original method, but were more pure. In addition a quantity of peptide 3 was also isolated and analyzed.

**Characterization of the peptides**

Peptide 4 from band 4 of the thin layer electrophoresis plate at pH 1.9 is Ser-Lys*-Asn. Amino acid analysis of peptide 4 after acid hydrolysis showed aspartate, serine and lysine in amounts approximately equal to the amount of PLS chromophore. Electrophoresis or thin layer chromatography of samples of peptide 4 that had been digested with aminopeptidase M showed that asparagine, not aspartic acid, was present in the peptide. The amino-terminal residue was serine. The peptide Ser-Lys*-Asn, where lys* is a lysine residue modified by PLS, corresponds to residues 257 through 259 in the protein and includes the active site lysine-258.

Peptide 2 was purified from band 2 of the pH 1.9 thin layer electrophoresis plate. The amino acid analysis of this peptide gave aspartic acid or asparagine, serine, glycine, leucine, phenylalanine, and lysine in amounts approximately equal to the amount of PLS chromophore. The amino terminus is serine. This peptide is therefore Ser-Lys*-Asn-Phe-Gly-Leu, residues 257 through 262 of the protein.

Peptide 3 is made up of aspartate or asparagine, serine, phenylalanine, and lysine. Phenylalanine is the amino terminal residue.
The peptide is therefore Phe-Ser-Lys*-Asn, residues 256 through 259 of the protein.

Band 1 from the electrophoresis at pH 1.9 most likely also contains a peptide or peptides that span lysine-258 modified by PLS. However this band contained only a small amount of the chromophore, so the peptides were never analyzed.

All the peptides containing the PLS chromophore that were analyzed include the active site lysine. This provides strong evidence that PLS binds to apoAAT in the same site as PLP.

The heterogeneity in the modified peptides is probably due to the lack of specificity in digestion by pepsin. However, prolonged digestion by pepsin could not convert all of the product to the smallest peptide, peptide 4.

Significant amounts of histidine were frequently indicated in the amino acid analyses of the PLS peptides, especially in those samples where the lysine content was less than expected. However, a sample of peptide 3 which showed histidine in the amino acid analysis, was tested with a reagent for histidine and showed none. The "histidine" in the amino acid analysis was possibly a modified lysine. A modified lysine would be retarded on the analyzer column if it were made more hydrophobic or if its pK were raised. Reaction of PLS with the ε-amino group of lysine could do either of these.

In the amino terminal analysis of each of the PLS peptides there was one fluorescent product which could not be identified as a dansylamino acid. I believe that this product may be due to the
reaction of dansyl chloride with the PLS product, possibly at the phenolic oxygen of the pyridoxyl moiety. Dansyl chloride reacts with the phenolic oxygen of tyrosine, and the 0-dansyl derivative has an orange fluorescence similar to that of the mysterious product from the PLS peptide. Furthermore, its location after thin layer chromatography on polyamide sheets is in the area of the dansyl derivatives of the large hydrophobic amino acids.

Interaction of the coenzyme analogs and apoenzyme

Yang et al. (1974) suggested that pyridoxal sulfate may act as a crosslinking reagent for two residues at the active site of aspartate aminotransferase, probably lysine-258 and some unknown group held by the enzyme in close proximity to the 4'-carbon of the coenzyme analog as it is held in a Schiff base linkage. I originally hypothesized that this unknown group was the sulfhydryl of cysteine-390, the syncatalytically modifiable sulfhydryl. This residue seemed to be the most likely candidate because it was already known to be located near the active site and because the model reaction of cysteine with PLS showed similar spectral properties to the enzymatic reaction. However, this hypothesis was disproved when PLS reacted with apoenzyme that had been blocked at cysteine-390 with cyanide, as shown by the spectral changes recorded in Figure 5. Furthermore, the modified peptides that were isolated were all single peptides containing lysine-258 but not cysteine-390, and the crystallographic structure of the native enzyme shows that cysteine-390 is not in the active site. Therefore, the unknown nucleophile
in the PLS reaction cannot be the sulfhydryl of cysteine-390.

Now that the peptides containing the PLS product have been identified, it is possible to speculate on the identity of the nucleophile and the nature of the product. If pyridoxal sulfate reacts to form a crosslink between lysine-258 and the side chain of another amino acid, and the cross-link is stable, then the nucleophile must be part of the same peptide since all three modified peptides were single peptides. The side chain of serine-257 or asparagine-259 are the only possibilities, since these two are the only other residues common to all three peptides. Of the two, the hydroxyl of the serine is more likely to participate in the reaction because it is a stronger nucleophile than the amide of asparagine. Serine reacts with PLS in solution at pH 9 to give a product with a narrow absorption band at 403 nm (24.8 kK) which has pronounced fine structure similar to the product of PLS with the apoenzyme (Yang et al., 1974). In the electron density map at 2.7 Å resolution of native aspartate aminotransferase it appears that residues 256 to 260 (phe-ser-lys-asn-phe) form a single turn of a helix, and that the side chains of all the residues involved are oriented downward, roughly antiparallel to the helical axis. Thus, the hydroxyl of serine-257 is located only a few Å from the Schiff base linkage between lysine-258 and pyridoxal phosphate. Very little reorientation either of the side chain of serine-257 or of pyridoxal sulfate within the coenzyme pocket of AAT would be necessary to allow the serine hydroxyl to react with the Schiff base.
This is the most attractive possibility for the identity of the unknown nucleophile.

Assuming that the unknown nucleophile is the hydroxyl of serine-257, it becomes possible to explain the properties of the chromophoric product of pyridoxal sulfate and apoAAT as shown in figure 28. Structure I represents peptide 4 at low pH, where it absorbs at 382 nm. This structure is fully protonated and unreactive. When the pH is raised to 8, the most acidic group, the phenolic oxygen, becomes deprotonated and forms a hydrogen bond with the Schiff base nitrogen, as in structure II. This may be the structure of the chromophore absorbing at 400 nm. In basic solution this structure could be hydrolyzed to a free serine residue and an amide of lysine-258 and deoxypyridoxic acid (structure III). This is seen as the irreversible loss of the absorbance at 400 nm on incubation at pH 8 or above. The 400-nm structure could be stable in the intact protein because of the close positioning of serine to the coenzyme or because the protein structure makes structure II inaccessible to hydroxide. The amide of structure III should be reasonably stable so that the chromophore remains attached to the peptide. Under rigorous conditions, such as those used to hydrolyze the peptides for amino acid analysis, the amide would be expected to give free lysine and 5'-deoxypyridoxic acid, structure IV. A non-specific protease, such as aminopeptidase M, may also cleave the amide so that very little or no modified lysine is detected in the digest on electrophoresis or thin-layer chromatography.
Figure 28  Possible reactions of the proposed pyridoxal chromophore involving lysine-258 and serine-257 of peptide 4. (See text for discussion.)
The major problem with this scheme is that it does not predict a structure for the putative modified lysine which runs on the amino acid analyzer column as histidine. Any of the structures in figure 26 would be expected to hydrolyze to lysine and deoxypyridoxic acid. Another difficulty is that Yang et al. (1974) found that the transition from the 386-nm chromophore to its form absorbing at 402 nm in the intact protein was not governed by a single pKa. The proposed analogous transition from structure I to structure II is a simple deprotonation.

Alternatively, the reaction may involve an amino acid side chain that is broken down in the course of the reaction leaving a fragment attached to the coenzyme. In this case an amino acid located some distance away from lysine-258 in the amino acid sequence would be involved in the reaction, but the formation of a crosslink would not be apparent from the isolated peptides. The guanido group of arginine and the imidazole of histidine are two possibilities for such a nucleophile. Both amino acids have been suggested in the active site from chemical modification studies, and both could be unstable if modified.

The X-ray crystallographic structure of the native enzyme shows no histidine residues near the Schiff base linkage between lysine and PLP, so this possibility must be discarded. On the other hand, there are several arginine residues near the coenzyme. Arginine-386 and arginine-292 apparently bind the two carboxyls of substrates, while arginine-266 interacts with the coenzyme phosphate. In the
2.7 Å electron density map it appears that arginine-386 could attack the PLS-lysine Schiff base if the coenzyme were a little crooked in the PLP site. This could result in the guanidino group breaking to leave a fragment attached to the PLS-lysine chromophore. Hydrolysis of this structure at pH 8 would be similar to the reaction in Figure 28, and would produce the same amide product as structure III in that scheme.

Another possibility is that the reaction simply involves water or hydroxide as the unknown nucleophile. The Schiff base of valine and PLS in solution at very high pH exhibits changes in its absorption spectrum that are similar to those seen in the reactions of PLS with apoAAT or β-substituted amines (A.E. Cahill, Department of Biochemistry, Iowa State University, unpublished results). The apoenzyme may be able to catalyze a similar reaction at a lower pH. However, such a structure would be expected to tautomerize rapidly to the amide, and from there it is not possible to write plausible chemical reactions to explain the complex conversions of the chromophore.

Whatever the nucleophile that reacts with the enzyme-PLS Schiff base, the apoenzyme must play an active role in its addition. In solution PLS reacts with a wide variety of amines and amino acids with nucleophilic substituents to produce spectral changes similar to those of the enzymatic reaction, but Schiff bases of PLS with valine or butylamine do not undergo further reactions at moderate pH. This indicates that the nucleophile must be held by the protein in close proximity to the Schiff base, and that the protein facilitates its addition to the Schiff base.
Miura and Metzler (1976) suggested that, because the reactions of apoAAT with pyridoxal sulfate and with the 5'-trans-carboxyethenyl analog of PLP were so similar, the unknown nucleophile was probably the same in both cases. The similarities in the peptides from the pepsin digestion of apoenzyme modified by the two coenzymes makes this all the more likely.

Aspartate aminotransferase is the only enzyme that is known to react with PLS in this way. However none of the other enzymes that have been tested are aminotransferases. This raises the question of whether the reaction is a quirk of this enzyme, or whether it is related to a step in the catalytic sequence that is unique to transamination. If the latter is the case, one would expect to see a similar reaction of PLS with other aminotransferases but not with vitamin B_{6}-dependent enzymes that catalyze other types of reactions. Dr. Retsu Miura is investigating the reactions of PLS and the carboxyethenyl analog with pig heart mitochondrial aspartate aminotransferase (personal communication). The results of his experiments ought to indicate whether the reaction is general among aminotransferases. Furthermore, the mitochondrial enzyme has an alanine residue rather than serine preceding the active site lysine (Morino and Watanabe, 1969; Kagamiyama et al., 1977; Barra et al., 1977). Therefore if PLS reacts with mitochondrial AAT as it does with the cytoplasmic isozyme, the hydroxyl of serine-257 can be fully ruled out as the unknown nucleophile.

Pyridoxal phosphate modifies either the mitochondrial or cytosolic
isoforms of malate dehydrogenase from pig heart in a reaction that
does some resemblance to the reaction of PLS with apoAAT (Wimmer
et al., 1975; Bleile et al., 1976). In the case of PLP and the malate
dehydrogenases the reaction is clearly biphasic. The first phase,
which is reversible, is the formation of a Schiff base between PLP and
a lysine residue in the enzyme active site. The second phase is irre­
versible and apparently involves the attack of a second nucleophilic
group of the protein on the PLP-lysine Schiff base to form an X-azoli­
dine similar to an intermediate in the proposed reaction of PLS and
apoAAT. The Schiff base of PLP and mitochondrial malate dehydrogenase
was reduced with sodium borohydride and digested with trypsin, and a
labeled hexapeptide was isolated and sequenced (Wimmer and Harrison,
1975). The X-azolidine product was stable when denatured in 6 M urea
and digested by trypsin, in contrast to the PLS-AAT product. A peptide
has been isolated whose amino acid content was consistent with the
hexapeptide of the reduced Schiff base with eight additional residues
(Gowan and Harrison, 1979). However, the fluorophore was destroyed in
the final purification step, electrophoresis at pH 3.7. Gowan con­
cluded that the unknown nucleophile was in a peptide contiguous with
the hexapeptide, and that it was most likely an arginine residue (L.K.
Gowan, Department of Biochemistry, Medical University of South Carolina,
personnal communication).

The reaction of PLP with malate dehydrogenase and the reaction
of PLS with apoAAT appear to be similar in some respects. Both
coenzymes react initially to form Schiff bases with specific lysine
residues of the proteins. In both cases an unknown nucleophile then attacks the Schiff base to form an X-azolidine structure. It is unknown whether phosphate is eliminated from the PLP-dehydrogenase X-azolidine, so this may not proceed through reactions analogous to the final steps of the PLS-AAT reaction sequence. Both reactions are irreversible in the absence of reduction, which is rare in reactions of pyridoxal compounds. However, while the PLS peptides from AAT are single peptides, the PLP peptide from malate dehydrogenase seems to be crosslinked although this peptide has not been characterized except by amino acid analysis.

It is interesting to note that the amino acid analysis of the X-azolidine peptide that Gowan isolated showed 0.6 equivalent of histidine. She stated (personal communication) that she originally believed this to be a contaminant, but finally concluded that there was really a histidine. In the light of the false indication of histidine in the amino acid analysis of the PLS peptides from apoAAT, this is intriguing. The "histidine" may be due instead to a PLP-modified lysine similar to the "histidine" of the PLS peptides. Of course, if PLP retains its phosphate group in the reaction with the malate dehydrogenase, it would hardly behave like the PLS product on the analyzer column.

The nature of the product of the reaction of pyridoxal sulfate with apoAAT needs further investigation. Its structure may become clear when the X-ray structure of the PLS-enzyme complex has been determined. The low-pH form of the complex has been crystallized
under the same conditions used to crystallize native AAT. The crystals have the same brilliant blue-white fluorescence under ultraviolet light as the enzyme-bound product in solution and the modified peptides (Figure 29).
Figure 29  A. Crystals of aspartate aminotransferase modified by pyridoxal sulfate.

Figure 29  B. The same crystals illuminated by UV light.
CONCLUSIONS

Aspartate aminotransferase has been crystallized by the vapor diffusion technique of McPherson (1976). In order to obtain heavy atom derivative crystals of the enzyme, the native enzyme and enzyme blocked at one or both of its surface sulfhydryls with small uncharged substituents was crystallized in the presence of one or two equivalents per monomer of methylmercuric chloride, p-chloromercuribenzoate or mercuric acetate. Although crystals formed with almost every combination of blocking group and mercury reagent, only one crystal was large enough for crystallography. A crystal of AAT blocked at both surface sulfhydryls with N-ethylmaleimide and crystallized with one equivalent of methylmercury showed one major and one minor mercury site in each monomer. The major site was cysteine-191 and the minor site was cysteine-82. These same two sites were occupied by methylmercury in a crystal of unmodified AAT. Mercuribenzoate binds to cysteine-82 and cysteine-45. Crystals of AAT containing two equivalents of methylmercury or mercuribenzoate have provided the two heavy atom derivatives used in calculating an electron density map at 2.7 Å resolution. Most of the polypeptide chain has been traced in this map. A crystal of apoenzyme was used to locate the binding site of the pyridoxal phosphate cofactor.

Pyridoxal sulfate reacts at the active site of apoAAT to modify lysine-258. Three peptic peptides from the enzyme modified by pyridoxal sulfate have been isolated and characterized by amino
acid analysis and N-terminal analysis. All include the active sequence ser-lys-asn-259. The identity of the second nucleophile involved in the reaction of PLS with apoAAT is still unknown. The isolated peptides were all single sequences with no apparent crosslink. From the X-ray structure of native AAT, it appears that the hydroxyl of serine-257 is in a position to participate as the second nucleophile in the reaction. Because it is close to the PLP-lysine Schiff base, it is most likely to react with the PLS-lysine Schiff base. If some distortion occurs in the active site of AAT when PLS binds, the guanidino group of arginine-386 could also be in a position to act as the unknown nucleophile. In this case, one must hypothesize that only a fragment of the arginine becomes incorporated into the PLS product since no arginine is indicated in the amino acid analyses of the isolated peptides. Hydroxide from the solvent may also be the nucleophile, but this possibility is less attractive than the others.

The 5'-trans-carboxyethenyl analog of PLP probably modifies the same active site peptides as PLS. The peptides modified with this analog behave similarly to the PLS peptides in the isolation procedures. However, peptides containing the carboxyethenyl analog were not isolated in sufficient yield to be characterized.
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