To the Graduate Council:

I am submitting herewith a dissertation written by Doo Sik Kim entitled "4-Aminobutyrate Aminotransferase." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Biochemistry.

Jorge E. Churchich, Major Professor

We have read this dissertation and recommend its acceptance:

Accepted for the Council:

Vice Chancellor
Graduate Studies and Research

4-AMINOBUTYRATE AMINOTRANSFERASE

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Doo Sik Kim

March 1983

DEDICATION

This dissertation is dedicated to the memory of my sister, Hea Ryung Kim, whose love remains warm and sweet in my mind.

ACKNOWLEDGEMENTS

The author is most grateful to Professor Jorge E. Churchich for the privilege and opportunity to work in his laboratory and wishes to thank him for the encouragement and advice based on his distinguished scientific insight and knowledge. I wish to thank all of the faculty members of my committee and Professor Wesley D. Wicks, chairman of the Biochemistry Department, for their stimulating discussions. Dr. Emil Schiltz of the University of Wurzburg must also be thanked for the advice and suggestion when the author visited his laboratory in the summer of 1982. I also am appreciative of all my fellow graduate students and Mr. Jack M. Williams who were very friendly with me in every situation.

I would like to show my deepest gratitude to my parents, Mr. and Mrs. Tae Won Kim, who stood by me and supported me during my entire education. Thanks also go to my parents-in-law, Mr. and Mrs. Tae Young Chung, for their encouragement.

My special thanks are due to Mr. Y. K. Kwon and Dr. J. H. Han for their moral support and warm friendship.

Additionally, I must thank Nancy Fogarty, Judy Lovely, and Linda Pilcher for their excellent typing work of this dissertation.

Finally, I wish to express my sincere thanks to my wife Sanghyun and my daughter Nayung who endured almost all the evenings as well as many weekends with great patience and love during the course of this research. Without their support, I could never have completed this work.

This work was supported by NIH Grant GM-27639 awarded to Dr. Jorge E. Churchich and by Graduate Teaching Assistantship awarded to the author from the University of Tennessee, Knoxville.

ABSTRACT

This work reports studies on 4-aminobutyrate aminotransferase (4-aminobutyrate: 2-oxoglutarate aminotransferase, EC 2.6.1.19), a key enzyme in the metabolism of neurotransmitter 4-aminobutyrate (GABA).

The mitochondrial enzyme 4-aminobutyrate aminotransferase from pig brain was purified 4000-fold by a combination of CM-Sephadex, DEAE-Sephadex and hydroxyapatite chromatography. This preparation, which migrates as a single band on 7.5% polyacrylamide gel electrophoresis, gives a specific activity of 20 units/mg protein. The enzyme is a dimeric protein with molecular weight of 100,000 made up of two subunits of identical molecular size. The purified holoenzyme contains 1 mol of pyridoxal-5-phosphate/mol of dimer. The dissociation constant for the cofactor molecule is 1 nM. Upon addition of pyridoxal-5-phosphate, a second cofactor molecule is bound to the holoenzyme with a dissociation constant of 3 μM. However, the enzyme has the same k_{cat} and K_m for 4-aminobutyrate before and after the binding of the second cofactor molecule.

Chemical modifications of the enzyme were accomplished by using gabaculine (5-amino-1, 3-cyclohexadiene carboxylic acid) and m-carboxyphenyl-pyridoxamine-5-phosphate as probes of the catalytic binding sites. The reaction of the enzyme with gabaculine was studied by observing changes in the absorption spectrum of the bound cofactor and by monitoring loss of catalytic activity. The enzyme is completely inactivated by gabaculine, but the dialyzed inactive sample containing 0.5 mol of gabaculine/mol dimer is fully reconstituted by addition of pyridoxal 5-phosphate. Stopped-flow kinetic studies reveal that gabaculine reacts with the cofactor bound to the aminotransferase with a

second-order rate constant of 2.5×10^3 M^{-1} s^{-1}. Fluorometric titrations of the apoprotein with m-carboxyphenyl-pyridoxamine-5-phosphate show the binding of two moles of the inhibitor/mole of enzyme. The binding process is reversible and the affinity of the apoprotein for the inhibitor is at least 10-fold higher than the affinity for the cofactor. It is postulated that the dimeric enzyme contains two potential active sites per dimer, but the binding site characterized by a weaker affinity constant for pyridoxal 5-phosphate becomes functional only after specific chemical modification of the molecule of cofactor tightly bound to the protein.

4-Aminobutyrate aminotransferase is inactivated by incubation with o-phthalaldehyde at pH 7.4. The reaction of 6 lysyl residues per dimer brings about 90% loss of the aminotransferase activity. The substrate 2-oxoglutarate at concentrations higher than the K_m = 0.1mM affords complete protection against the inactivation. Several lines of experimental evidence indicate that o-phthalaldehyde reacts with lysyl residues other than those involved in the binding of pyridoxal-5-P.

It is postulated that the carboxyl groups of 2-oxoglutarate interact with positively charged lysyl residues located at the catalytic site.

4-Aminobutyrate aminotransferase is inactivated by preincubation with bispyridoxal-5-P (mixing molar ratio, 20:1) at pH 7.0. The reaction with bispyridoxal-5-P under pseudo-first order conditions proceeds at a slow rate (k_{obs} = 0.03 min^{-1}).

The extent of chemical modification was determined by measuring the spectroscopic properties of P-pyridoxyl and P-pyridoxine chromophores formed after reduction of the enzyme reacted with P^1P^2-bis (5'-pyridoxal) diphosphate. Reduction with $NaBH_4$ results in the incorporation of approximately 2.1 P-

pyridoxyl residues/dimer. Thus, the blocking of 2 lysyl residues/dimer is needed for inactivation of the aminotransferase. The time course of inactivation is significantly affected by variations in the pH of the reaction mixtures. Plots of k_{obs} versus pH indicate the reaction of the bifunctional reagent with lysyl residues characterized by low pK values (pK = 7.3).

The substrate 2-oxoglutarate (10 mM) affords complete protection against inactivation, whereas pyridoxal-5-P failed to prevent the inactivation of the enzyme by bispyridoxal-5-P. It is postulated that binding of 2-oxoglutarate to lysyl residues is the major factor contributing to stabilization of the catalytic site. Several lines of experimental evidence indicate that inactivation of the aminotransferase cannot be related to dissociation of the cofactor from the catalytic site. The bifunctional reagent bispyridoxal-5-P blocks lysyl residues other than those involved in the binding of the cofactor.

4-Aminobutyrate aminotransferase is cleaved by trypsin yielding enzymatically active species which can be separated from the split off peptides by gel filtration. The shortened enzyme derivative gives one band (95,000 MW) on polyacrylamide gradient gel electrophoresis. Changes in protein conformation induced by tryptic digestion were studied by fluorescence spectroscopy. The native enzyme tagged with 5-iodoacetamidofluorescein yields a rotational relaxation time of 106 nanoseconds, whereas the trypsin digested enzyme gives a rotational relaxation time of 33 nanoseconds. The decrease in rotational relaxation time is attributed to flexibility of the polypeptide chain with enhanced rotational freedom of the probe covalently linked to one thiol group. The reactivity of sulfhydryl groups toward 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) is also affected by the trypsin cleavage. More SH groups (2.5/dimer) become reactive toward DTNB as result of trypsin digestion. Local

conformational fluctuations are induced as result of the tryptic cleavage, but the catalytic sites remain intact.

The peptides released from 4-aminobutyrate aminotransferase by the trypsin digestion were analyzed on finger-print, and their amino acid composition was determined. The release of peptides of 5,500 molecular weight can account for the difference in molecular weights between native and trypsin treated enzymes.

These results suggest that the multiple forms of 4-aminobutyrate aminotransferase are the products of proteolytic digestion.

The N-terminal sequence of 16 amino acid residues of 4-aminobutyrate aminotransferase was determined by solid-phase technique. The PTH-amino acids produced in the peptide sequencing indicated that the two subunits are homologous peptide chains.

The tryptic peptide of 20 amino acids containing phosphopyridoxyl-lysine was isolated and purified by a combination of gel filtration and ion exchange chromatgraphy. The sequence of this fragment determined by automated Edman degradation was compared with those of cytosolic and mitochondrial aspartate aminotransferase.

TABLE OF CONTENTS

CHAPTER PAGE

LIST OF TABLES

LIST OF FIGURES

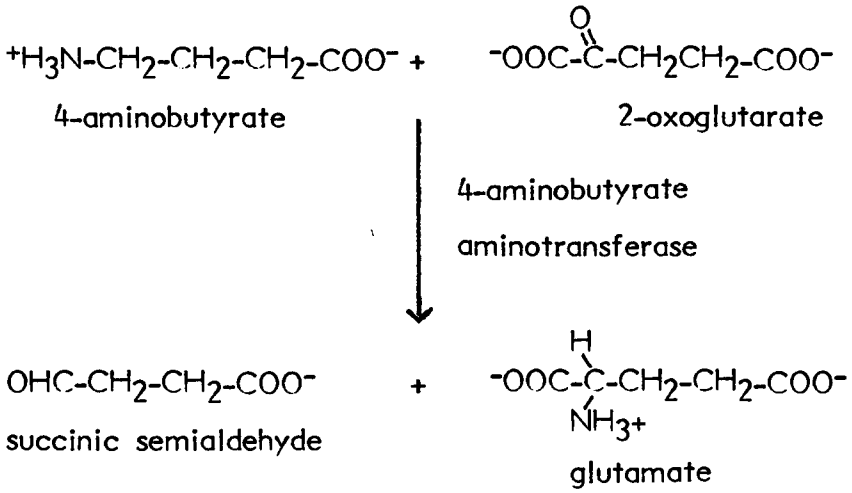
FIGURE PAGE

FIGURE PAGE

INTRODUCTION

Since it is now well established that 4-aminobutyrate serves as an important constituent in the intermediary metabolism of brain (1) and a major inhibitory neurotransmitter in many invertebrate systems or in the vertebrate central nervous system (2,3,4), it has become considerabley important to obtain more detailed knowledge about the enzymes directly involved in the metabolsim of 4-aminobutyrate, i.e., 4-aminobutyrate aminotransferase.

The mitochondrial enzyme 4-aminobutyrate aminotransferase (EC 2.6.1.19) is a typical pyridoxal-5-phosphate-dependent transaminase that catalyzes the transamination of 4-aminobutyrate with the enzyme-bound cofactor pyridoxal-5-phosphate to yield succinic semialdehyde and the pyridoxamine form of the cofactor. The oxidized cofactor is reformed by transamination with 2-oxoglutarate to yield glutamate and pyridoxal-5-phosphate.



4-Aminobutyrate aminotransferase has been isolated and purified to homogeneity from brain tissues of different mammals including mouse, rat, rabbit, pig and human (5,6,7,8,9,10). More recently the first example for the

crystallization of 4-aminobutyrate aminotransferase isolated from Pseudomonas was reported by Yonaha and Toyama (11) although the Pseudomonas transaminase (MW 178,000) has a larger molecular size than the mammalian brain enzyme (MW 100,000) and their absorption spectroscopic properties are different from each other.

It is generally agreed that 4-aminobutyrate aminotransferase purified from different brain sources have almost identical molecular weights of around 100,000 and consist of two equal size of subunits. However their physico-chemical and kinetic properties, such as K_m values for 4-aminobutyrate and 2-oxoglutarate, vary from one species to another, which may suggest that the enzyme is strictly species specific (12).

The existence of multiple forms of 4-aminobutyrate aminotransferase has been reported in pig brain (6), guinea pig brain (13) and in pig liver (14). The functions of multiple forms of the enzyme were related to the regulation of 4-aminobutyrate metabolism. However, no clear experimental evidence for the speculation has been demonstrated so far. Some kinetic and physical properties of liver 4-aminobutyrate aminotransferase have been compared to those of brain enzyme, and no significant differences have been observed except for their sedimentation coefficients (14). On the other hand, comparative studies of certain structural features of rat and human brain 4-aminobutyrate aminotransferase reveal that the two enzymes, which have approximately same molecular weight with identical N-terminus amino acid (Ile), exhibit considerable difference in the tryptic finger-prints and in the peptides produced by cyanogen bromide cleavage (15). In addition, the K_m values for 4-aminobutyrate are different, the human enzyme having four times greater affinity for the amino substrate. A series of branched chain fatty acids, including n-dipropylacetate,

which are structural analogues of 4-aminobutyrate and inhibit rat brain 4-aminobutyrate aminotransferase, are less powerful inhibitors for the human enzyme (15).

Inhibition studies for 4-aminobutyrate aminotransferase have been carried out by several groups. Rando and Bangerter (16,17,18) have shown that gabaculine (5-amino-1,3-cyclohexadiene carboxylic acid), which is a naturally occuring amino acid, irreversibly inhibits the transaminase by specifically derivatizing the enzyme-bound cofactor pyridoxal-5-phosphate. The mechanism of inhibition involves the catalytic turnover of gabaculine to give an activated intermediate which reacts with pyridoxal-5-phosphate to yield m-carboxyphenyl pyridoxamine phosphate by spontaneous aromatization (18). It was demonstrated by Burnett et. al. (19) that the reaction of only 0.45 mol gabaculine per tetramer is needed to inactivate Pseudomonas ω -amino acid: pyruvate transaminase indicating the negative cooperativity between subunits. Other substrate analogues such as 2,4-diaminobutyric acid, aminooxyacetic acid and ethanolamine-O-sulfate were also introduced as active site-directed inhibitors for rat brain (20) and rabbit brain 4-aminobutyrate aminotransferase (21).

Recent studies by Churchich and Moses (22) in our laboratory have indicated the presence of non-equivalent binding sites of pig brain 4-aminobutyrate aminotransferase which are characterized by two different affinities for the cofactor molecule. Based on their experimental evidence, it was postulated that the catalytic binding site characterized by a weaker affinity for pyridoxal-5-phosphate becomes functional only after specific chemcial modification of the cofactor molecule tightly bound to the protein. Moses and Churchich (23) also showed that the pig brain enzyme is inactivated by incubation with 5,5'-dithiobis-2-nitrobenzoic acid, and the reaction of one

thiol group per dimer brought about almost complete loss of the aminotransferase activity. However, little information is available about the amino acid residues which are critically connected with catalytic function of 4-aminobutyrate aminotransferase.

Immunochemical studies have demonstrated that the antibody to purified mouse brain 4-aminobutyrate aminotransferase cross-reacts with the enzyme from the following species: human, calf, rat, guinea pig, rabbit and frog whereas the transaminase from birds, fishes and bacteria did not cross-react (24).

4-Aminobutyrate aminotransferase seems to be present in a variety of tissues with the highest activity in brain (25,26). Employing a histochemical technique in which NADH produced in the reaction of 4-aminobutyrate aminotransferase and succinic semialdehyde dehydrogenase reduces nitroblue-tetrazolium, several investigators have found the transaminase in both neurons and glial cells with the majority of the activity present in neurons (27,28,29,30,31).

Brain 4-aminobutyrate aminotransferase is known to be associated with mitochondria (32,33,34,35), and on the basis of experiments in which mitochondria were treated with Triton X-100, it has been shown that the enzyme is located in the mitochondrial matrix (33). More recent studies demonstrated that the transaminase is associated with the inner membrane of brain mitochondria (36).

CHAPTER I

GABACULINE AND m-CARBOXYPHENYL-PYRIDOXAMINE-5-PHOSPHATE PROBES OF THE CATALYTIC BINDING SITES OF 4-AMINOBUTYRATE AMINOTRANSFERASE

Gabaculine, 5-amino-1-3-cyclohexadiene carboxylic acid, is a naturally occuring amino acid (37) which inhibits pyridoxal-5-P linked 4-aminobutyrate aminotransferase (38). The mechanism of the inactivation process involves transamination of the inhibitor followed by spontaneous aromatization which generates m-carboxyphenyl-pyridoxamine-5-P (Cppp) (18).

The tight binding of the stable adduct to the active site of the enzyme results in an apparent irreversible inhibition.

More recently, Burnett et al. (19) have studied the inhibition of Pseudomonas ω -amino acid pyruvate transaminase by labelled gabaculine and they have shown that less than 1 mol of labelled gabaculine/tetramer is needed to inhibit the aminotransferase.

This interesting finding, which indicates negative cooperativity between subunits, prompted us to investigate the catalytic behavior of mammalian 4-aminobutyrate aminotransferase reacted with gabaculine.

Since gabaculine belongs to a class of inhibitors which are substrates of their target enzymes, it can be used to investigate the relationship between pyridoxal-5-P content and the catalytic activity expressed by mammalian 4-aminobutyrate aminotransferase.

The spectroscopic properties, absorption and fluorescence, of m-carboxyphenyl-pyridoxamine-5-P bound to the enzyme can be used to determine

the stoichiometry of binding, the origin of the apparent irreversible binding process, and the microenvironment of the catalytic sites.

I. EXPERIMENTAL

Purification of 4-Aminobutyrate Aminotransferase

Unless otherwise specified, the purification steps were performed at 4°C and all the buffers contained 0.1mM and 1mM 2-mercaptoethanol without the addition of pyridoxal-5-P.

Pig brains were obtained from East Tennessee Packing Co. The brains were removed as soon as possible after slaughter and preparation was begun within 1 hour. A Waring Blender was used to prepare a 25% (w/v) homogenate in a solution of 10mM potassium phosphate (pH 7) containing 1mM 2-oxoglutarate. The resulting homogenate was adjusted to pH 5.5 by the slow addition of 1M acetic acid, heated to 50°C for 5 minutes and, after cooling to 4°C, centrifuged at 10,000 g for 30 minutes. The precipitate was discarded and the supernatant was treated with $(NH_4)_2SO_4$. The precipitate obtained at 40 to 75% saturation was dissolved in 0.01 M sodium acetate buffer (pH 5.7) and dialyzed overnight against several changes of the same buffer (Buffer I). It was then applied to a column (5 x 80 cm) of CM-Sephadex equilibrated with buffer I and washed with 500 ml of the equilibration buffer. The enzyme was eluted by using a linear gradient made with the equilibration buffer (500 ml) and the same volume of 0.3 M sodium acetate buffer (pH 5.7). At the end of the gradient elution, the column was washed with 250 ml of 0.3 M sodium acetate buffer (pH 5.7) to ensure complete elution of the enzyme. The enzyme is eluted at an acetate concentration of 0.28 M.

The active fractions were combined and concentrated using an Amicon concentrator. The protein solution was dialyzed against 0.01 M potassium phosphate (pH 7.4) (Buffer II) and applied to a DEAE-Sephadex column (2.6 x 30 cm) previously equilibrated with Buffer II. The column was then washed with 100 ml of the equilibrating buffer and eluted by using a linear gradient made with the equililbration buffer (Buffer II), 250 ml, and the same volume of 0.2 M potassium phosphate (pH 7.4). The enzyme is eluted at a phosphate concentration of 0.05 M.

The active fractions were combined and concentrated to a final volume of 50 ml. The protein was then dialyzed against 0.01 M potassium phosphate (pH 7) (EDTA was omitted) (Buffer III) and applied to a hydroxyapatite column (0.9 x 20 cm) previously equilibrated with Buffer III. The enzyme was eluted by using a linear gradient made with Buffer III (50 ml) and the same volume of 0.35 M potassium phosphate (pH 7). The enzyme is eluted at a phosphate concentration of 0.28 M. The active fractions were combined and dialyzed against 0.1M potassium phosphate (pH 7.4). A scheme of the purification is included in Table 1.1.

The enzyme has a specific activity of 20 units/mg of protein and a molecular weight of 100,000 (9). Brain pyridoxine-5-P oxidase was purified by a method already described (39). The rate of formation of pyridoxal-5-P was measured by following the increase in absorbance at 388 nm (39).

TABLE I.I

PURIFICATION OF 4-AMINOBUTYRATE AMINOTRANSFERASE FROM PIG BRAIN

The purification of 4-aminobutyrate aminotransferase was made from 5 kg of pig brain tissue, net weight.

Treatment	Volume	Protein	Specific Activity	Yield
	ml	mg/ml	units/mg*	%
Homogenate	12,000	40	0.005	100
Supernatant after 50°C	6,000	6.2	0.022	34
$(NH_4)_2SO_4$ (45-75% SATN)	800	10.4	0.035	12
CM-Sephadex Fraction (0.01-0.3M acetate, pH 5.7)	200	9.9	0.094	7.8
DEAE-Sephadex Fraction (0.01-0.2M phosphate pH 7.4)	50	3.4	0.8	5.7
Hydroxyapatite (0.01-0.35 M phosphate, pH 7)	5	I	20	4.2

*A unit of enzyme activity is defined as that amount of enzyme which produces I μ mol/min of succinic semialdehyde at 25°C.

Determination of Bound Pyridoxal-5-P

Protein concentration was determined by the colorimetric method of Lowry et al., (40) and by absorbance measurements at 280nm. A sample of apoenzyme at a concentration of 1 mg/ml gives an absorbance of 1.2 at 280 nm (1 cm light path). The molar extinction coefficient of the protein at 280 nm is 1.2×10^5 $M^{-1}cm^{-1}$. The pyridoxal-5-P content of the purified aminotransferase was determined by the method of Wada and Snell (41). The molar extinction coefficient of bound pyridoxal-5-P is 1.8×10^4 M^{-1} cm^{-1} at 415 nm. The same value is obtained by dissolving the holoenzyme in 0.1M NaOH and determining the pyridoxal-5-P content using a molar extinction coefficient of 6,600 at 388 nm for pyridoxal-5-P.

Enzymatic Assays

Two methods were used in the assay of 4-aminobutyrate aminotransferase activity.

Method 1: This enzyme assay is based on fluorimetric measurements of the condensation product of cyclohexane 1-3-dione with succinic semialdehyde (9). The reagent was a solution containing 0.25g of cyclohexane 1-3-dione, 10g ammonium acetate, 5 ml glacial acetic acid in 100 ml of water. The substrate solution contained 20mM 4-aminobutyrate and 10mM 2-oxoglutarate adjusted to pH 8.4 with NaOH. The substrate solution (5 ml) is incubated with the enzyme at 37°C. Aliquots (0.5 ml) withdrawn from the incubation mixture at several time intervals were mixed with 0.4 ml of the reagents solution, heated for 15 minutes in a water bath at 60°C, diluted to 3 ml by addition of water and the fluorescence intensity recorded at 460nm with 365nm

excitation. A standard curve of succinic semialdehyde reacted with cyclohexane 1-3 dione was run in parallel. This method permits the detection of succinic semialdehyde concentrations lower than 0.1 μM without interference by the reagent cyclohexane 1-3-dione.

Method II: A coupled assay system consisting of two purified enzymes, i.e., 4-aminobutyrate aminotransferase and succinic semialdehyde dehydrogenase was used to study the catalytic conversion of 4-aminobutyrate into succinic semialdehyde.

Enzymatic assays were performed in 0.1M sodium pyrophosphate (pH 8.4) containing 5mM NAD^+, 20mM 4-aminobutyrate and 10mM 2-oxoglutarate. The progress of the reaction was followed by monitoring changes in absorbance at 340nm due to the reduction of NAD^+. The coupled assay system effectively measures the rate of transamination when the concentration of succinic semialdehyde dehydrogenase is at least five fold greater than the concentration of 4-aminobutyrate aminotransferase. Succinic semialdehyde dehydrogenase was purified from pig brain by a method already described (42).

Initial rate measurements were carried out by monitoring the change in absorbance at 340nm for at least 2 minutes, good straight lines being generally obtained. The results were plotted as $1/v$ against $1/[S]$ for one substrate, with different fixed concentrations of the other substrate, to give two sets of parallel lines indicating a ping-pong mechanism. These lines were analyzed for their intercept values. The values of Vmax determined in this manner were converted into k_{obs} by dividing by the molarity of the enzyme used. The plot of $1/k_{obs}$ against $1/[S]$ gives k_{cat}.

Resolution of 4-Aminobutyrate Aminotransferase

The enzyme (20μM) in 0.1M potassium phosphate (pH 7.4) containing 1mM 2-mercaptoethanol was allowed to react with 40mM 4-aminobutyrate at 25°C for 10 minutes. The pH of the solution was decreased to pH 6 by addition of KH_2PO_4 to a final concentration of 0.5M and kept at 4°C for 30 minutes. Then it was dialyzed against 1M potassium phosphate monobasic containing 1 mM 2-mercaptoethanol for 1 hour at 4°C, followed by dialysis against 0.1M potassium phosphate (pH 7.4) or 0.05 M HCl-triethanolamine buffer (pH 7.4) at 4°C for 3 hours. The resulting apoenzyme is inactive, but regains more than 90% of the original enzymatic activity after addition of pyridoxal-5-P.

Synthesis of m-Carboxyphenyl-pyridoxamine-5-P

A methanol solution of dipotassium pyridoxal-5-P (1 mmole) was added in small portion to 1 mmole of the potassium salt of DL-gabaculine in dry methanol (Total volume 15 ml). The reaction was allowed to proceed at 60°C for 1 hour. The solution was treated with 0.02 M $NaBH_4$ at 4°C, the mixture was filtered, acidified with acetic acid and evaporated. The precipitate was dissolved in water and chromatographed on ion exchange resin, Amberlite XE-64 (Column 2 x 50 cm) with water as the eluting solvent. Fractions absorbing at 330 nm and 290 nm were collected and freeze dried. The purity of the compound was tested by thin layer chromatography on silica gel plates using the solvent system butanol-water-acetic acid (2/2/1). The pure compound gave a single spot on thin layer chromatography. Its spectroscopic properties (absorption and fluorescence) are identical to those reported in the literature (17).

Polyacrylamide Gel Electrophoresis

The enzyme preparations were examined by polyacrylamide gel electrophoresis at pH 8.3 according to the original procedure of Davis (43). Electrophoresis was conducted at 25°C in a Buchler analytical disc electrophoresis apparatus regulated at 4mA/tube. Protein bands were detected by staining with naphtol blue black and destaining with 7% acetic acid. Sodium dodecyl sulphate electrophoresis on polyacrylamide gels containing 7.5% polyacrylamide, 1% 2-mercaptoethanol and 0.1% sodium dodecyl sulphate was performed according to the method of Weber and Osborn (44). Electrophoresis was conducted as above but at 8 mA/tube. Protein bands were detected by staining with Coomassie blue dye for one hour and subsequently destained overnight in a solution containing 10% methanol and 7% acetic acid in water. The four protein bands used as standards in the determination of molecular weight were bovine serum albumin, ovalbumin, pepsin and lysozyme.

Spectroscopy

Fluorescence spectra were recorded in a precision spectrofluorimeter equipped with two Bausch and Lomb monochromators. The slits of the monochromators were set to give a band width of 3nm. Spectrophotometric measurements were carried out on either a Cary model 15 or an Aminco DW-2 double beam spectrophotometer. The latter, equipped with a stopped-flow accessory, was used to follow the course of faster reactions. Under pseudo-first-order conditions, k_{obs} was determined using equation [1]

$$A_t - A_\infty = (A_o - A_\infty)\, e^{-k_{obs} \cdot t} \qquad [1]$$

where A_t, A_o and A_∞ are the absorbances at time t, o and ∞, respectively.

Ultracentrifugation

Sedimentation velocity experiments were conducted in the Spinco Model E analytical ultracentrifuge at constant temperatures in the range of 8 - 10°C. For sedimentation velocity studies, the ultracentrifuge was operated at 56,100 RPM and the sedimentation constants reported were corrected for the density and viscosity of water at 20°C. Samples of purified 4-aminobutyrate aminotransferase at concentrations varying between 2 mg and 8 mg/ml in 0.1M phosphate buffer (pH 7.4) containing 1mM 2-mercaptoethanol were studied in the analytical ultracentrifuge.

Materials

CM-Sephadex C-50, and DEAE-Sephadex were purchased from Pharmacia; Pyridoxal-5-P, Pyridoxamine-5-P, 4-aminobutyrate, 2-oxoglutarate, succinic semialdehyde from Sigma, and cyclohexane-1-3-dione from Aldrich Chemical Company.

Hydroxyapatite was prepared by the method of Tiselius et al. (45), DL-gabaculine was purchased form Boehringer.

II. RESULTS

The enzyme 4-aminobutyrate aminotransferase, purified by the method developed in our laboratory, was found to be homogeneous since a single protein and activity band was detected by polyacrylamide gel electrophoresis. The enzyme migrates as a single peak in the analytical ultracentrifuge with a sedimentation coefficient of $s_{20,w} = 5.4S$.

The subunit molecular weight, as determined by sodium dodecylsulphate polyacrylamide gel electrophoresis, corresponds to a monomeric unit of 50,000

molecular weight. The enzyme contains 1 mol of tightly bound cofactor per dimer, and the cofactor exhibits two absorption bands centered at around 330 and 415 nm at pH 5.6 or at pH 8.2. Fig. 1.1 shows the results obtained at pH 7.4.

Incubation of the enzyme with five fold molar excess of DL-gabaculine results in complete inhibition of catalytic activity. The reaction is accompanied by a change in the absorption spectrum of the bound cofactor; the absorbance at 415nm decreases simultaneously with an increase in absorbance at 340 nm (Fig. 1.1). At the concentrations of enzyme and DL-gabaculine used in these experiments, i.e. five fold molar excess of gabaculine, the reaction reaches completion in less than 5 minutes.

The inhibited enzyme is not reactivated in the least by dialysis against 0.1M phosphate (pH 7.4) containing 1mM 2-mercaptoethanol. Most surprisingly, the absorption spectrum of the dialyzed inactive enzyme exhibited an intense absorption band at 340 nm which might be attributed to retention of m-carboxyphenyl-pyridoxamine-5-P (Fig. 1.1).

Further support for the presence of m-carboxyphenyl-pyridoxamine-P in the dialyzed inactive enzyme was derived from fluorescence measurements. The fluorescence emitted by the adduct is significantly quenched upon binding to the protein; but dissociation of m-carboxyphenyl-pyridoxamine-5-P from the protein is attained by denaturation of the modified enzyme with 5M guanidinium-HCl as shown by the results of Fig 1.2.

Judging from the intensity of the emission band obtained upon excitation at 315 nm, it appears that a fraction of the derivatized coenzyme remains bound to the protein.

For a quantitative determination of m-carboxyphenyl-pyridoxamine-5-P bound to the enzyme, the emission spectra of samples of modified enzyme in

Figure 1.1

Absorption spectra of 4-aminobutyrate aminotransferase (15 μ M) (1), 4-aminobutyrate aminotransferase (15 μM) reacted with DL-gabaculine (80μM) (2) and dialyzed against 0.1 M phosphate buffer (pH 7.4) for 8 hours (3).

Absoprtion spectra of 4-aminobutyrate aminotransferase (15 μ M) preincubated with pyridoxal-5-P (80 μM) (4) and reacted with DL-gabaculine (80 μM) (5) at pH 7.4.

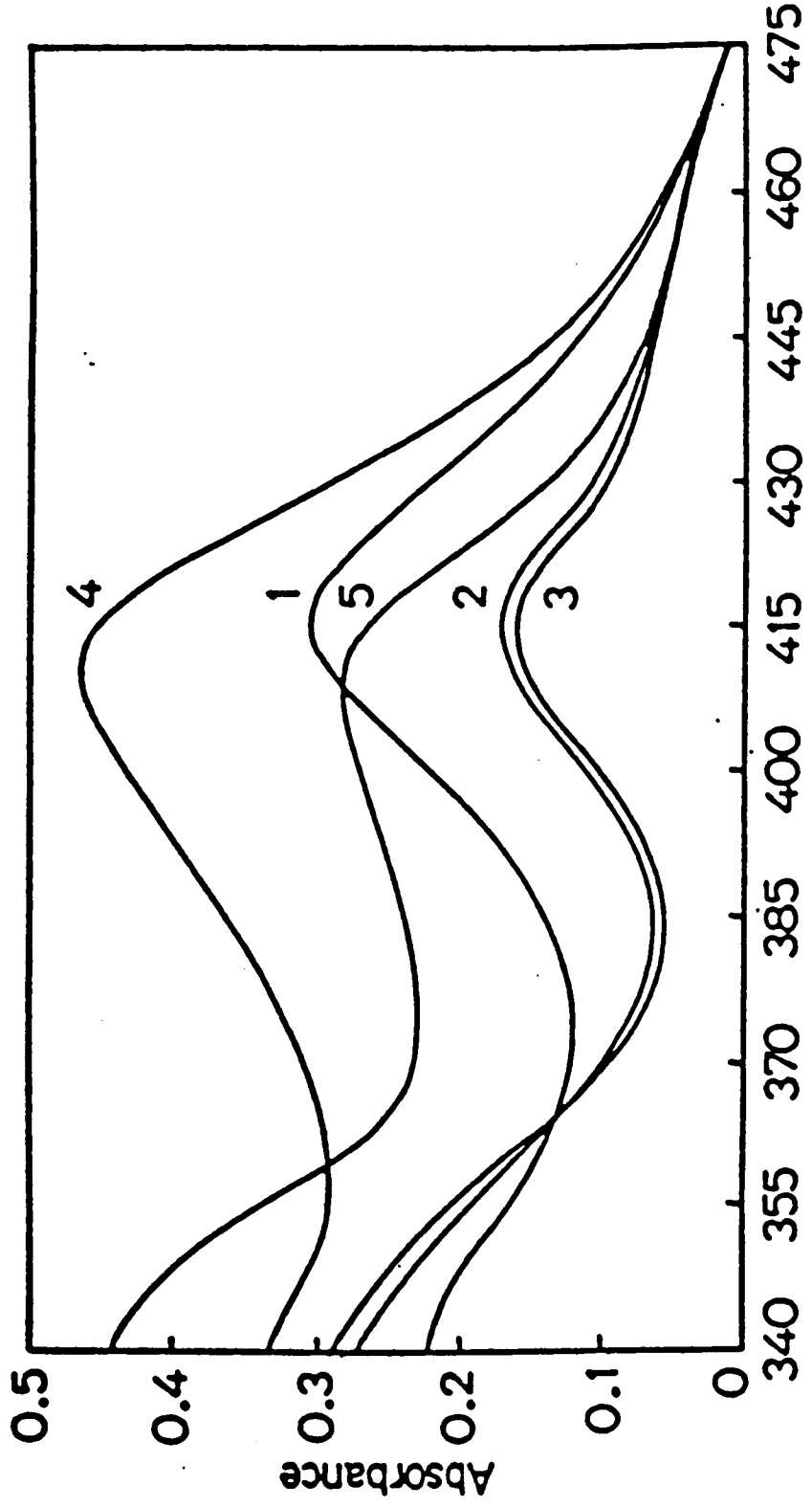

Absorbance
0.5
0.4
0.3
0.2
0.1
0
340
355
370
385
400
415
430
445
460
475
Wavelength (nm)
4
1
5
2
3

Figure 1.1

Figure 1.2

Emission spectra of 4-aminobutyrate aminotransferase reacted with 4-fold molar excess of DL-gabaculine and dialyzed against 0.1M phosphate buffer (pH 7.4) for 8 hours. Spectra recorded in the absence (1) and presence of 5M guanidinium-HCl (2) at a protein concentration of 5 μM. The emission spectra of m-carboxyphenyl-pyridoxamine-5-P (5 μM) in 5M guanidinium-HCl (3) is included. Excitation wavelength 315 nm.

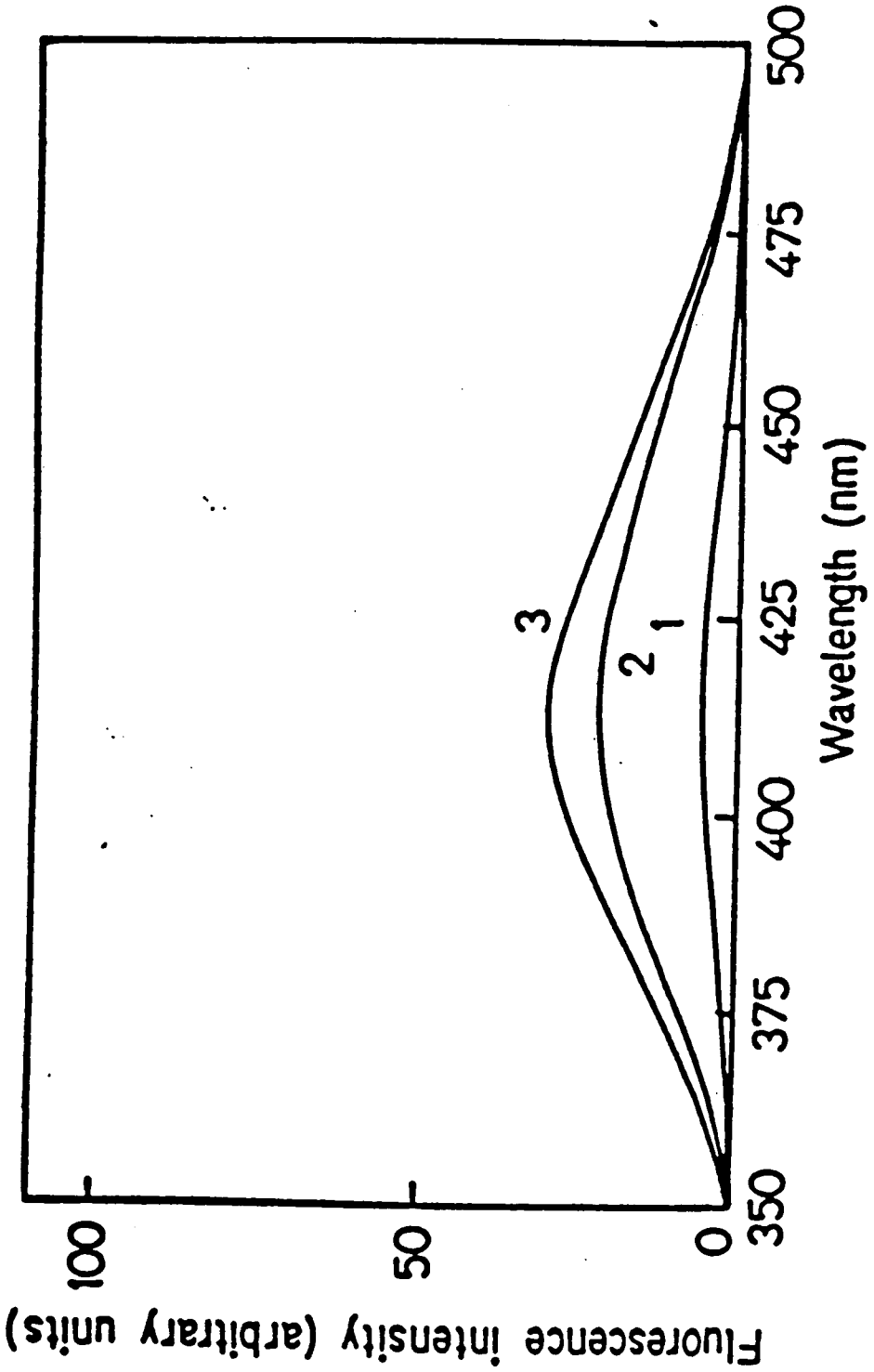

Fluorescence intensity (arbitrary units)
100
50
0
350
375
400
425
450
475
500
Wavelength (nm)
1
2
3

Figure 1.2

5M guanidinium-HCl were compared to the emission spectra of samples of m-carboxyphenyl-pyridoxamine-5-P in 5M guanidinium-HCl. The fluorescence emitted at 415 nm, excited at 310, 315, and 320 nm, is strictly proportional to the concentration of m-carboxyphenyl-pyridoxamine-5-P over the concentration range 4×10^{-6}M - 2×10^{-5}M.

The presence of derivatized coenzyme bound to the protein (0.5 mol/dimer) does not prevent full recovery of catalytic activity upon subsequent preincubation of the enzyme with 10-fold molar excess of pyridoxal-5-P (Table 1.2). As may be seen from the results included in Table 1.2, the V_{max} and K_m values of the derivatized enzyme reconstituted by addition of pyridoxal-5-P are, within experimental error, identical to those of the native enzyme.

To test whether the enzyme saturated with pyridoxal-5-P contains more than one molecule of cofactor involved in catalysis, the reaction of the enzyme with DL-gabaculine was allowed to proceed for five minutes and the absorption spectra immediately recorded over the wavelength range 330-475 nm.

The results of these measurements indicate a decrease in absorbance at 415 nm (Fig. 1.1) with a concomitant loss of catalytic activity (Table 1.2). Extensive dialysis of the derivatized enzymes did not restore their catalytic function, but the fraction of m-carboxyphenyl-pyridoxamine-5-P bound to the protein decreases as the dialysis process is prolonged (Table 1.2).

Although addition of 10 fold molar excess of pyridoxal-5-P restores some of the catalytic activity, the degree of recovery of aminotransferase activity depends upon the fraction of m-carboxyphenyl-pyridoxamine-5-P bound to the protein.

TABLE 1.2

CATALYTIC PROPERTIES OF NATIVE AND GABACULINE DERIVATIZED 4-AMINOBUTYRATE AMINOTRANSFERASE

Sample	Mol Adduct/Mol Enzyme	Vmax	Kcat (sec^{-1})	K_M^1 (mM)	K_M^2 (mM)
Enzyme (1 mol PLP/Dimer)	0	100	9.6	0.9	0.10
Enzyme (Saturated with PLP)	0	100	9.6	0.9	0.10
Derivatized Enzyme (I)[a]	0.5	100	9.6	0.8	0.10
Derivatized Enzyme (II)[b]	1.4	20	—	—	—
Derivatized Enzyme (II)[c]	1.2	30	—	—	—

a: Native enzyme (10 μM) reacted with DL-gabaculine (40μM), dialyzed for 4 hours at 4°C against 0.1M phosphate (pH 7.4) containing 1mM 2-mercaptoethanol and reconstituted by incubation with pyridoxal-5-P (100 μM) for 1 hour at 25°C.

b and c: Native enzyme (10 μM) preincubated with pyridoxal-5-P (50 μM), reacted with DL-gabaculine (40μ M), dialyzed for 4 (b) and 10(c) hours against 0.1M phosphate (pH 7.4) containing 1mM 2-mercaptoethanol and reconstituted by incubation with pyridoxal-5-P (100 μM) for 1 hour at 25°C.

Kinetics of the Reaction

The kinetics of the reaction of 4-aminobutyrate aminotransferase with DL-gabaculine was examined at increasing molar ratios of inhibitor to enzyme. The extent and rate of the reaction was monitored by following spectral changes at 415 nm.

When the reaction was studied in the presence of large excess of DL-gabaculine (0.8 mM), under pseudo first order conditions, it was found that the profile of the reactivity curve is monophasic (Fig. 1.3). Similar profiles were observed at lower concentrations of inhibitor when the reaction was monitored at 25°C in 0.1 M phosphate buffer (pH 7.4). A plot of k_{obs} versus inhibitor concentrations yields a second order rate constant of $2.5 \times 10^3 M^{-1} sec^{-1}$. When the enzyme saturated with pyridoxal-5-P was allowed to react with 30 fold molar excess of DL-gabaculine, and the reaction monitored at 415 nm, it was observed that the pseudo first order rate constant $k_{obs} = 0.55$ sec^{-1} is, within experimental error, identical to that determined for the native enzyme.

As shown in Fig. 1.3, the profile of the reactivity curve is indeed monophasic, though more than one molecule of pyridoxal-5-P per dimer has reacted with DL-gabaculine.

In marked contrast to the bound cofactor, free pyridoxal-5-P does not react with gabaculine to form m-carboxyphenyl-pyridoxamine-5-P under the experimental conditions chosen for the kinetic measurements. Reaction of free pyridoxal-5-P with DL-gabaculine takes place at higher temperatures, 80°C, after incubation times much longer than 3 seconds (17).

Figure 1.3

Graphical analysis of reaction of 4-aminobutyrate aminotransferase (5.2 μM) with 0.15 mM (1), 0.26 mM (2) and 0.8 mM (3) DL-gabaculline at pH 7.4 (25°C).

Reaction of 4-aminobutyrate aminotransferase (5.2 μM), preincubated with pyridoxal-5-P (15μ M), with 0.15 mM DL-gabaculine (4) at pH 7.4, 25°C. The total change in absorbance at 415 nm ($A_o - A_{oo}$) for the native and pyridoxal-5-P saturated enzymes are 0.053 and 0.086, respectively.

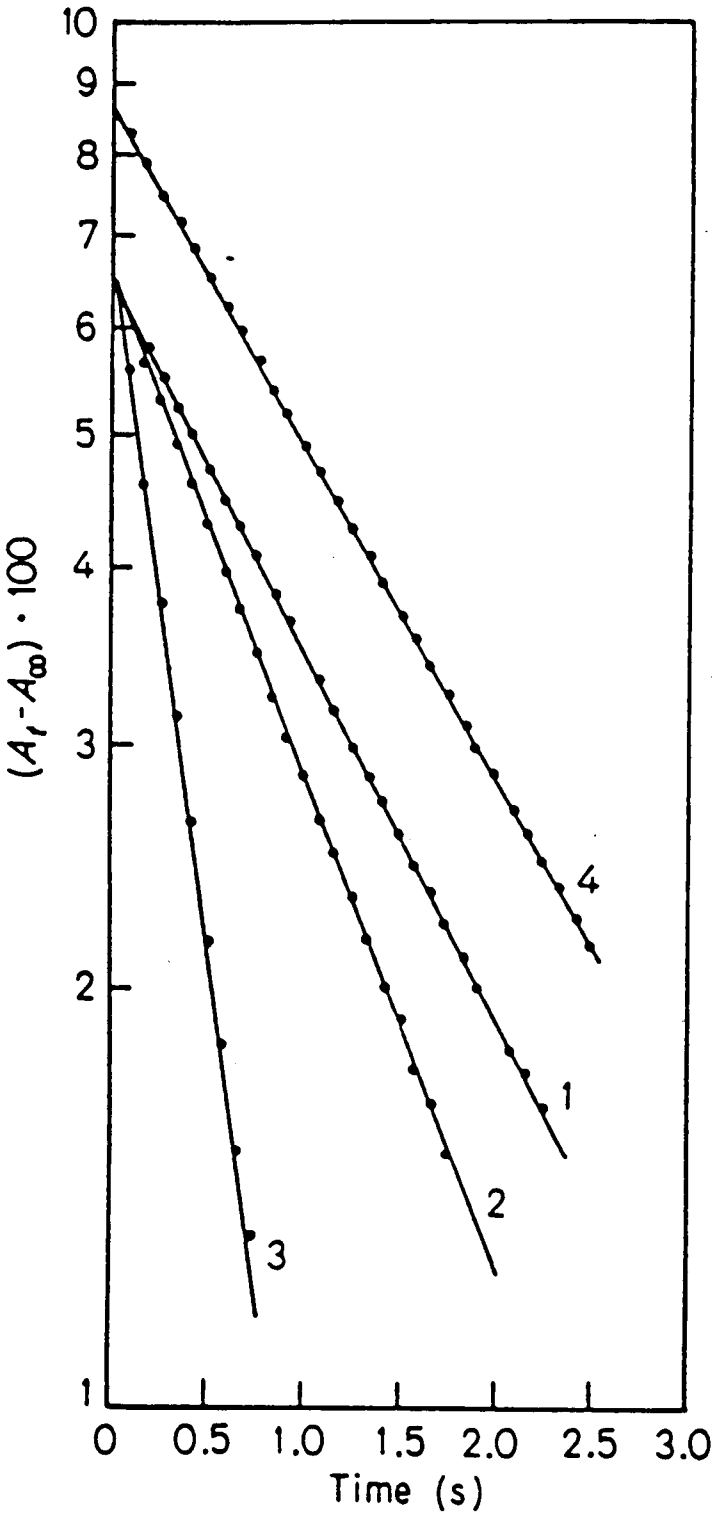


Figure 1.3

Reversible Binding of m-Carboxyphenyl-pyridoxamine-5-P

In order to obtain more information about the nature of the binding process when m-carboxyphenyl-pyridoxamine-5-P interacts with the active site of the aminotransferase, we decided to investigate the binding of the adduct to the apoprotein using enzymatic and fluorescence methods.

The apoprotein is inhibited by m-carboxyphenyl-pyridoxamine-5-P, which is an excellent substrate for pyridoxine -5-P oxidase.

At pH 8, the K_m for m-carboxyphenyl-pyridoxamine-5-P is 1 μM and the specific activity of the oxidase is 120 units/mg of enzyme at 25°C. With pyridoxine-5-P as a substrate, the K_m is 13 μM and the specific activity 70 units/mg of enzyme (39).

Since the reaction catalyzed by the oxidase is essentially irreversible, it is likely that a shift in the equilibrium

apoprotein-m-carboxyphenyl-pyridoxamine-5-P $\rightleftharpoons$ apoprotein + m-carboxyphenyl-pyridoxamine-5-P

will take place as a result of the oxidation of m-carboxyphenyl-pyridoxamine-5-P to pyridoxal-5-P (PLP) according to the following equation:

m-carboxyphenyl-pyridoxamine-5-P + O_2 + H_2O $\xrightarrow{\text{oxidase}}$ pyridoxal-5-P + NH_4^+ + H_2O_2

If the binding of the adduct to the apoenzyme is reversible, then one should be able to detect an increase in aminotransferase activity as a consequence of pyridoxal-5-P binding to vacant catalytic sites. The results of these experiments are given in Fig. 1.4, where it may be seen that full recovery of catalytic activity is attained in the presence of the oxidase.

m-Carboxyphenyl-pyridoxamine-5-P displays a maximum of emission at around 410 nm upon excitation at 300, 310, 315, and 330 nm, whereas

Figure 1.4

Kinetics of the reconstitution of 4-aminobutyrate aminotransferase activity after incubation of the apoenzyme (10 μM) with pyridoxal-5-P (0.1 mM) (0), apoenzyme (10 μ M) + m-carboxyphenyl-pyridoxamine-5-P (60 μ M) (■), and apoenzyme (10 μM) + m-carboxyphenyl-pyridoxamine-5-P (60 μM) oxidase (1μ M) (●) in 0.1M phosphate buffer (pH 7.4) at 4°C. Effect of preincubation at 4°C on the enzymatic activity of 4-aminobutyrate aminotransferase (▲). Recovery of enzymatic activity was monitored by using a coupled assay system (Method II, Experimental).

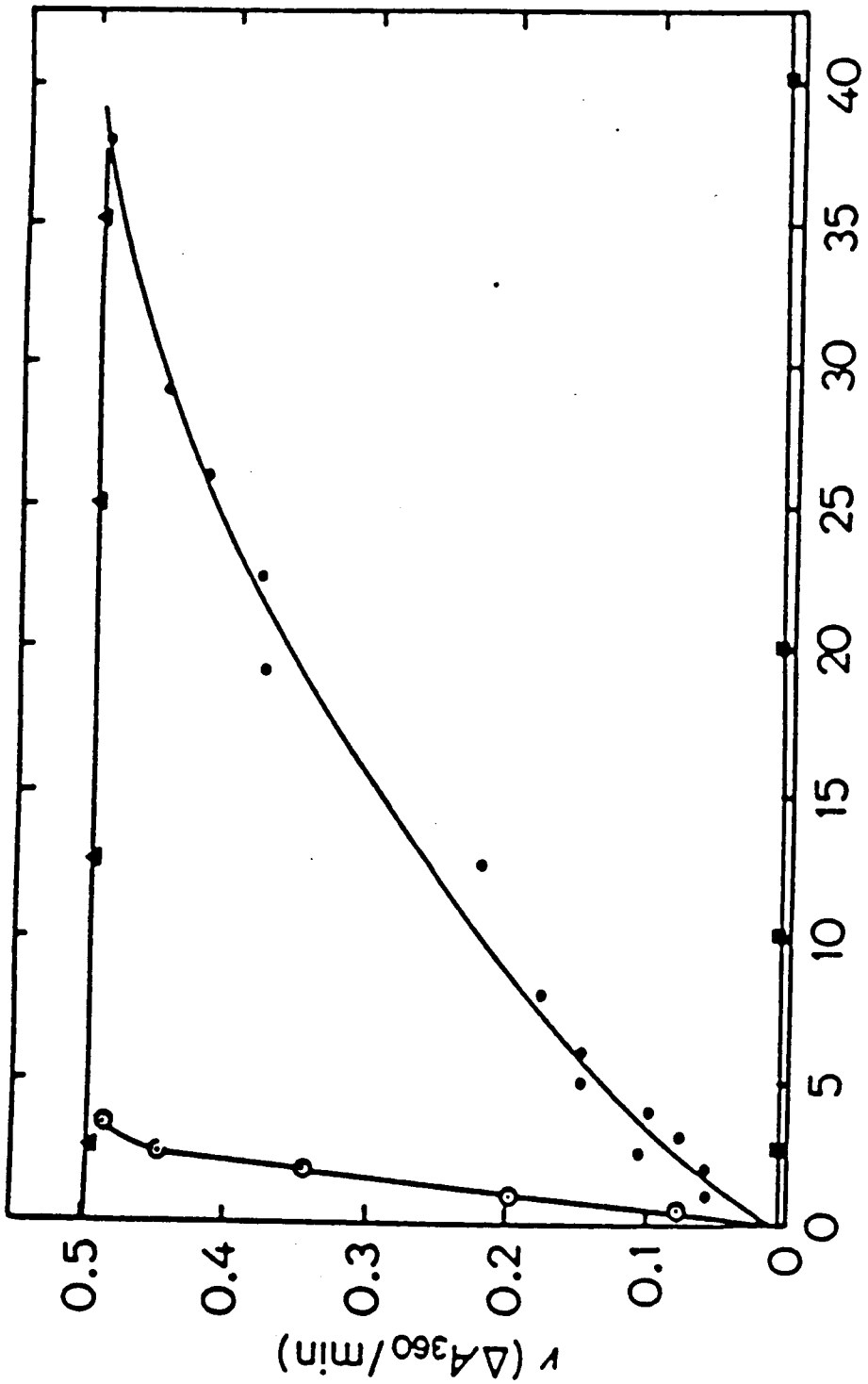


Figure 1.4

pyridoxamine-5-P exhibits a band centered at around 390 nm when excited over the wavelength range 300-330 nm (Fig 1.5). The emission band of m-carboxyphenyl-pyridoxamine-5-P coincides with that of m-aminobenzoic acid, though the quantum fluorescence yield of the latter compound is higher than that of the adduct (Fig 1.5).

The fluorescence emitted by m-carboxyphenyl-pyridoxamine-5-P is quenched upon formation of a complex with the apoprotein, and this quenching effect was used to determine the number of catalytic binding sites of the enzyme.

To this end, the fluorescence of samples containing fixed concentration of apoprotein (2 μ M) and varying concentrations of m-carboxyphenyl-pyridoxamine-5-P were recorded and compared to the fluorescence emitted by known concentrations of free m-carboxyphenyl-pyridoxamine-5-P.

The ratio of the fluorescence intensities in the absence (Fo) and presence of protein (F) varies as a function of increasing concentrations of inhibitor (Fig. 1.6). A maximum quenching effect was measured at a molar mixing ratio of apoenzyme to adduct of approximately 1:2, indicating that saturation of two binding sites has taken place.

Determination of the Inhibition Constant

Accurate measurements of the equilibrium dissociation constant (K_d) for binding of m-carboxyphenyl-pyridoxamine-5-P are difficult to perform by spectroscopic methods because the determination of K_d would require fluorometric titrations at protein concentrations lower than 0.1μ M. For this reason, the inhibition constant (K_i) was determined by enzymatic methods.

Figure 1.5

Emission spectra of m-aminobenzoic acid (1), pyridoxamine-5-P (2) and m-carboxyphenyl-pyridoxamine-5-P (3), excited at 315 nm. All the samples have the same absorbance (A = 0.1) at the exciting wavelength. Temperature 25°C.

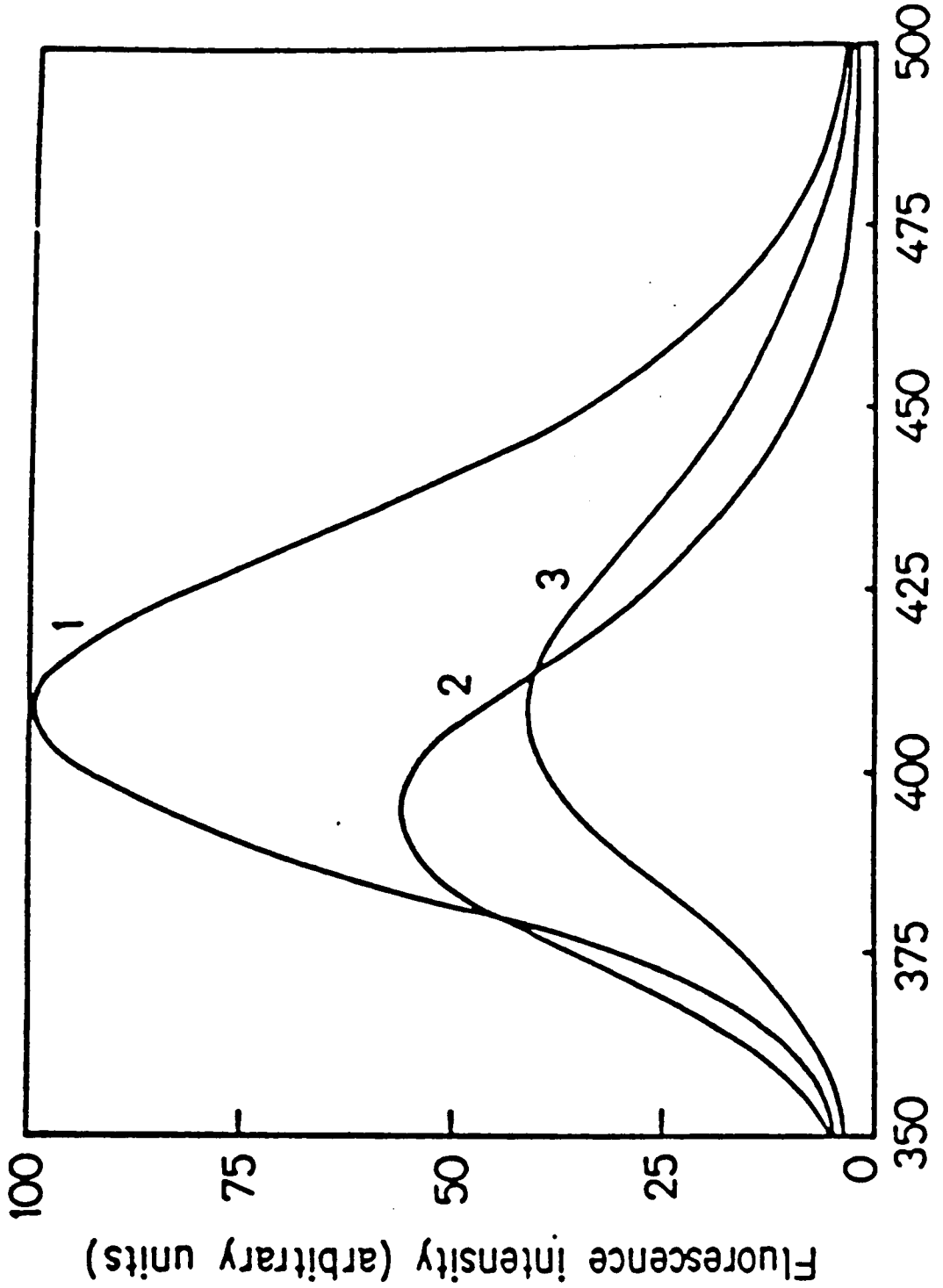

Fluorescence intensity (arbitrary units)
100
75
50
25
0
350
375
400
425
450
475
500
Wavelength (nm)
1
2
3

Figure 1.5

Figure 1.6

(A) Changes in fluorescence intensity ratio (F_o/F) at 420 nm (excitation 315 nm) upon addition of varying concentrations of m-carboxyphenyl-pyridoxamine-5-P to a fixed concentration of apoenzyme (2 μ M) in 0.1M phosphate buffer (pH 7.4). (B) Effect of m-carboxyphenyl-pyridoxamine-5-P on the activation of resolved 4-aminobutyrate aminotransferase by pyridoxal-5-P in 0.1M phosphate buffer (pH 7.4). Apoenzyme (1 μM) was mixed with pyridoxal-5-P (0.1 mM) and inhibitor at the indicated molar ratios to pyridoxal-5-P. After 1-h incubation at 25°C, samples were assayed for aminotransferase activity. A K_i value for m-carboxyphenyl-pyridoxamine-5-P of 1 nM was calculated from the slope of the straight line with a value of 10^{-8} M for the affinity of pyridoxal-5-P for the apoprotein.

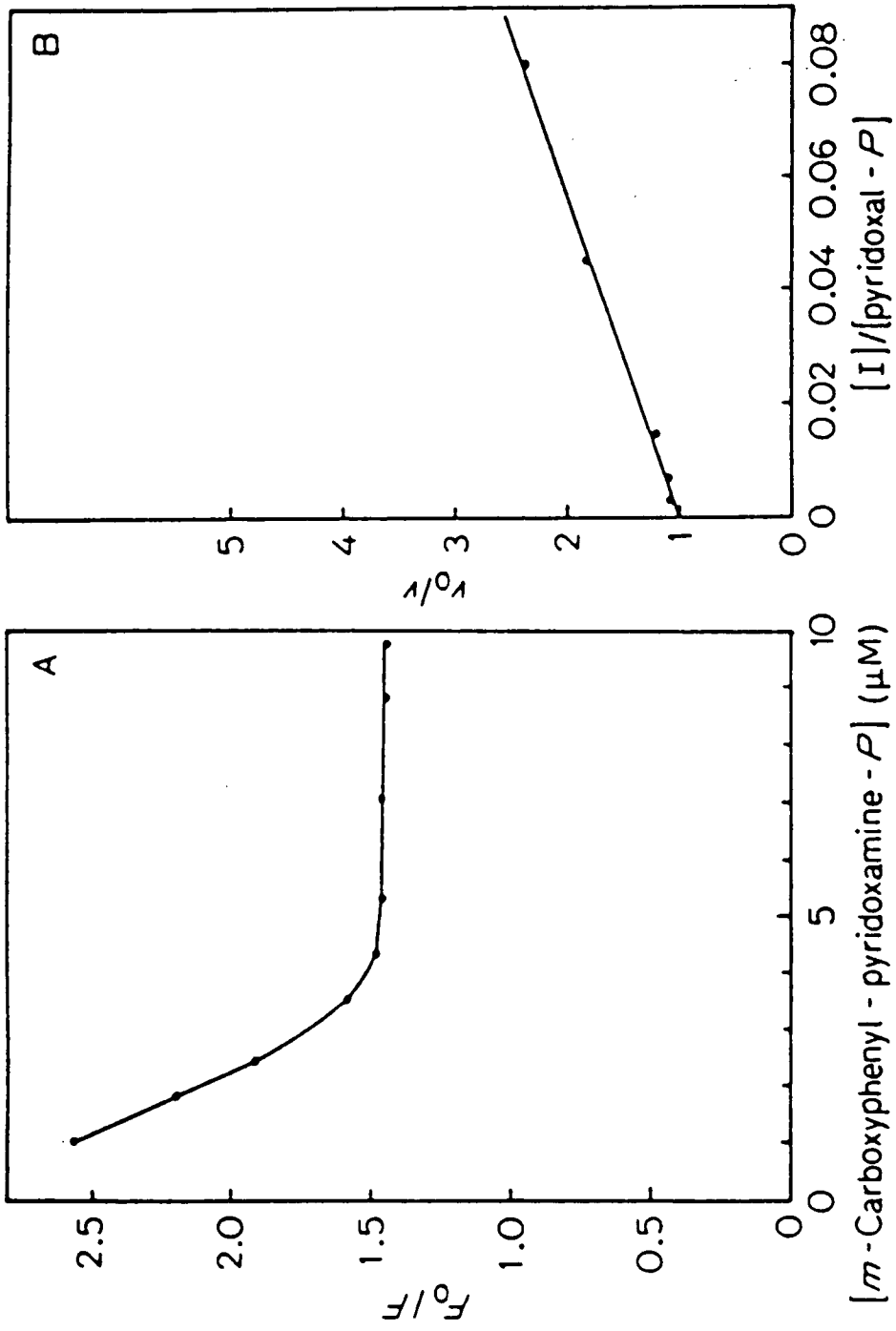

A
B
F_o/F
v_o/v
[m-Carboxyphenyl - pyridoxamine - P] (μM)
[I]/[pyridoxal - P]
0
0.5
1.0
1.5
2.0
2.5
5
10
0.02
0.04
0.06
0.08
1
2
3
4
5

Figure 1.6

The apoprotein was always added to mixtures containing a fixed concentration of pyridoxal-5-P and variable concentrations of inhibitor. After equilibrium between apoprotein, inhibitor and cofactor has been achieved, the samples were assayed for aminotransferase activity using a coupled assay system.

The plot of v_o/v versus [I]/ [pyridoxal-5-P] is given in Fig 1.6, where it may be seen that the K_i obtained from the slope of the straight line is approximately 10 fold lower than the dissociation constant of pyridoxal-5-P bound to the enzyme.

III. DISCUSSION

In the present work the relationship between catalytic activity expressed by 4-aminobutyrate aminotransferase and the extent of chemical modification of pyridoxal-5-P groups reacted with the suicide substrate gabaculine was examined.

The mechanism of inactivation of the aminotransferase involves transamination of gabaculine followed by spontaneous aromatization which generates m-carboxyphenyl-pyridoxamine-5-P (18). Despite the tight binding of m-carboxyphenyl-pyridoxamine-5-P to the catalytic sites of the enzyme, the binding process is reversible as demonstrated by the formation of enzymatically active species after coupling with an irreversible reaction which converts m-carboxyphenyl-pyridoxamine-5-P to pyridoxal-5-P. The slow rate of recovery of 4-aminobutyrate aminotransferase activity observed in the presence of the oxidase reflects the slow rate of dissociation of the inhibitor from the catalytic sites of the aminotransferase. In fact, using the K_i determined by enzymatic

methods ($K_i = 10^{-9}M$) and the second order rate constant $k_2 = 2.5 \times 10^{3}M^{-1}sec^{-1}$ for the reaction of gabaculine with the native enzyme, one can determine a first order rate constant ($k_1 = 2.5 \times 10^{-6}sec^{-1}$) for the dissociation of the inhibitor. The magnitude of this first order rate constant is compatible with the observed long delay of recovery of enzymatic activity (35 hours). Hence, the apparent irreversible binding of m-carboxyphenyl-pyridoxamine-5-P is mainly due to its slow rate of dissociation from the catalytic sites.

When the reaction of the enzyme containing 1 mol of pyridoxal-5-P/dimer was studied in the presence of 4-fold molar excess of gabaculine, it was found that binding of m-carboxyphenyl-pyridoxamine-5-P (0.5 mol/dimer) did not influence the ability of the inactive enzyme to bind pyridoxal-5-P and regain full catalytic activity. On the other hand, derivatization of the enzyme saturated with pyridoxal-5-P by reaction with gabaculine resulted in the incorporation of more than 1 mol of m-carboxyphenyl-pyridoxamine-5-P/dimer with concommitant losses in catalytic activity. These results can be interpreted in terms of a simple model which assumes a) the presence of two potential reactive sites per dimeric structure and b) both sites bind tightly the inhibitor m-carboxyphenyl-pyridoxamine-5-P. The presence of two binding sites on the apoprotein for m-carboxyphenyl-pyridoxamine-5-P was detected by means of fluorescence spectroscopy, whereas the results of inhibition studies indicated that the affinity of m-carboxyphenyl-pyridoxamine-5-P for the apoprotein is at least ten times greater than the affinity of the cofactor for the catalytic site.

Although the results obtained with the suicide substrate gabaculine are consistent with the idea that two potential active sites per dimer are reactive, it should be noted that the catalytic behavior of the native enzyme saturated with pyridoxal-5-P is very unusual.

A comparison of the k_{cat} and K_m values of native and pyridoxal-5-P saturated enzymes reveals that binding of additional molecules of pyridoxal-5-P has no effect on the kinetic parameters. Thus, maximum catalytic activity is already attained when one molecule of pyridoxal-5-P is bound per dimer, suggesting that only one reactive center per dimer is catalytically active. Apparently, the second reactive center of the enzyme becomes functional only after chemical modification of the molecule of cofactor which is tightly bound to the dimeric structure.

In this respect, 4-aminobutyrate aminotransferase differs from aspartate aminotransferase which is known to exhibit maximum catalytic efficiency when two binding sites are saturated with pyridoxal-5-P (46).

CHAPTER II

4-AMINOBUTYRATE AMINOTRANSFERASE, THE REACTION OF LYSINE RESIDUES CONNECTED WITH ENZYMATIC ACTIVITY

The enzyme 4-aminobutyrate aminotransferase from pig brain has been purified and characterized in two laboratories (6,23). The enzyme is a dimeric protein made up of two subunits of identical molecular weight (6,23). The mechanism of action is described by the ping-pong mechanism for two substrate enzymes (8,47).

Little information is available about the amino acid residues responsible for binding the substrates and the amino acid residues which participate in the catalytic step. Recent studies have demonstrated that the blocking of one SH group per dimer leads to irreversible loss of the catalytic activity, but the rate of inactivation is not influenced by the presence of substrate pairs in the reaction mixture (23).

The aims of the present investigation are two-fold. Firstly, to study the reaction of o-phthalaldehyde with lysine residues of the protein, and, secondly, to investigate the interaction of the substrate with charged amino acid residues at the catalytic site.

I. EXPERIMENTAL

Purification of the Enzyme

The purification of the enzyme was performed by a method developed in our laboratory (23). All the purification steps were performed at 4°C and all the buffers contained 1mM 2-mercaptoethanol without the addition of pyridoxal-5-P.

Enzymatic Assay

A coupled assay system consisting of two purified enzymes, i.e., 4-aminobutyrate aminotransferase and succinic semialdehyde dehydrogenase was used to monitor the catalytic conversion of 4-aminobutyrate into succinic semialdehyde. Enzymatic assays were performed in 0.1M sodium pyrophosphate (pH 8.4) containing 5mM NAD^+, 20mM 4-aminobutyrate and 10mM 2-oxoglutarate. The progress of the reaction was followed by monitoring changes in absorbance at 340 nm due to the reduction of NAD^+. Succinic semialdehyde dehydrogenase was purified from pig brain by a method already described (42).

Lysine Modification

The reaction of o-phthalaldehyde with the enzyme was performed at 25°C. The enzyme (4 μM) was preincubated with o-phthalaldehyde in 0.1M sodium phosphate buffer (pH 7.4), containing 1mM 2-mercaptoethanol, at 25°C. Aliquots (10 μl) withdrawn from the incubation mixture were assayed for aminotransferase activity.

A fresh solution of o-phthalaldehyde in 0.1M sodium phosphate (pH 7.4), containing 1mM 2-mercaptoethanol, was prepared before the reaction with the enzyme (48). The degree of labelling of the enzyme reacted with o-

phthalaldehyde was determined from the absorption spectrum using a molar extinction coefficient of $7.3 \times 10^3 M^{-1} cm^{-1}$ at 340 nm.

Absorption spectra were recorded on a Cary model 15 spectrophotometer and emission spectra in a precision spectrofluorimeter equipped with two Bausch and Lomb monochromators. The amino acid composition of native and modified with o-phthalaldehyde was dialyzed against water, lyophylized and then dissolved in 6N HCl. The hydrolysis tubes were flushed with nitrogen, evacuated, and maintained at 110°C for 24 hours.

II. RESULTS

The enzyme 4-aminobutyrate aminotransferase reacts with o-phthalaldehyde, which is a reagent for ε-amino groups of lysine residues in proteins (42). The effect of addition of o-phthalaldehyde on the enzymatic activity of 4-aminobutyrate aminotransferase was investigated by preincubating the enzyme with increasing concentrations of o-phthalaldehyde at a temperature of 25°C in 0.1M phosphate buffer (pH 7.4) containing 1mM 2-mercaptoethanol. Inactivation of the enzyme was attained when the protein was preincubated with excess o-phthalaldehyde (mixing molar ratio of reagent, enzyme 15:1) at 25°C for 10 minutes.

Fig. 2.1 shows the time course of reaction of the enzyme with o-phthalaldehyde at 25°C. Under this set of experimental conditions, the loss of catalytic activity was completely prevented by the addition of 10mM of the substrate 2-oxoglutarate prior to the reaction with o-phthalaldehyde. It should be noted that addition of pyridoxal-5-P prior to reaction with o-phthaladehyde has no effect on the rate and extent of inactiviation.

Figure 2.1

4-Aminobutyrate aminotransferase (4 μM) was incubated with 15 fold molar excess of o-phthalaldehyde at 25°C (pH 7.4). Results obtained in the absence (●) and presence (O) of 2-oxoglutarate (10mM) in the incubation mixture. The decay in enzymatic activity of a sample of enzyme (4 μM) preincubated with pyridoxal-5-P (1mM) prior to the addition of o-phthalaldehyde is given in the figure (□).

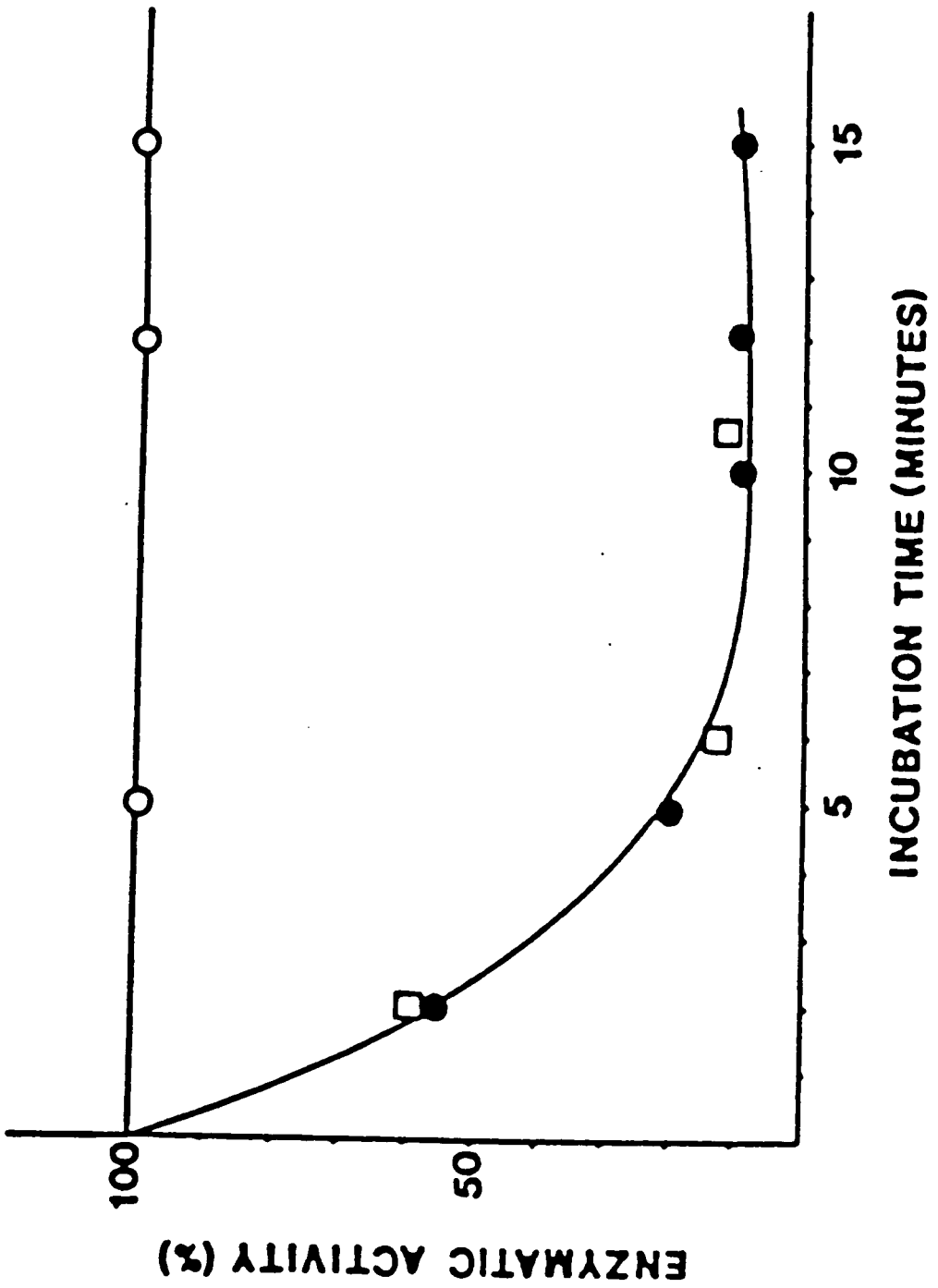


Figure 2.1

Spectroscopic Studies

The extent of the reaction of o-phthalaldehyde with ε-amino groups of lysine residues of the enzyme is easily determined by measuring either the increase in absorbance at 335 nm or the increase in fluorescence at 450 nm (42). Free o-phthalaldehyde does not absorb at 335 nm (48) and it does not exhibit any fluorescence over the spectral range 350 to 500 nm. Experiments designed to correlate changes in enzymatic activity with the number of o-phthalaldehyde molecules reacted, revealed that 80% loss of catalytic activity is achieved after the reaction of approximately 6 mol of o-phthalaldehyde/mol of enzyme (Fig. 2.2). When the absorption spectra of native and modified enzymes were recorded over the spectral range 300-500 nm, it was observed that the modified enzyme reacted with o-phthalaldehyde preserves the absorption band centered at 415 nm, which is due to the presence of pyridoxal-5-P bound to the protein (Fig. 2.3). Hence, irreversible inactivation of the enzyme upon reaction with o-phthalaldehyde cannot be ascribed to dissociation of the cofactor from the catalytic site.

The absorption spectrum of the modified enzyme resembles that of α-benzyloxycarbonyl-lysine and polylysine reacted with o-phthalaldehyde (Fig. 2.3). Samples of polyarginine preincubated with o-phthalaldehyde at pH 7.4 failed to show any absorption band at 335 nm as indicated in Fig. 2.3. This finding is interpreted to mean that the arginyl residues do not react with o-phthalaldehyde.

Further support for this contention was derived from amino acid analysis of samples of native and modified enzymes subjected to acid hydrolysis.

Figure 2.2

Inhibition of 4-aminobutyrate aminotransferase by o-phthalaldehyde. The enzyme (4 μM) was preincubated with 8 μM (1), 12 μM (2), 16 μM (3), 30 μ M (4) and 50 μ M (5) of o-phthalaldehyde at 25°C (pH 7.4) for 15 minutes. The absorbance at 340 nm was immediately recorded, and aliquots (10 μ l) were assayed for aminotransferase activity. Results obtained with the holoenzyme (●) and with the apoenzyme (o). The samples of apoenzyme reacted with o-phthalaldehyde were preincubated with pyridoxal-5-P (0.1mM) for 30 minutes prior to enzymatic assays.

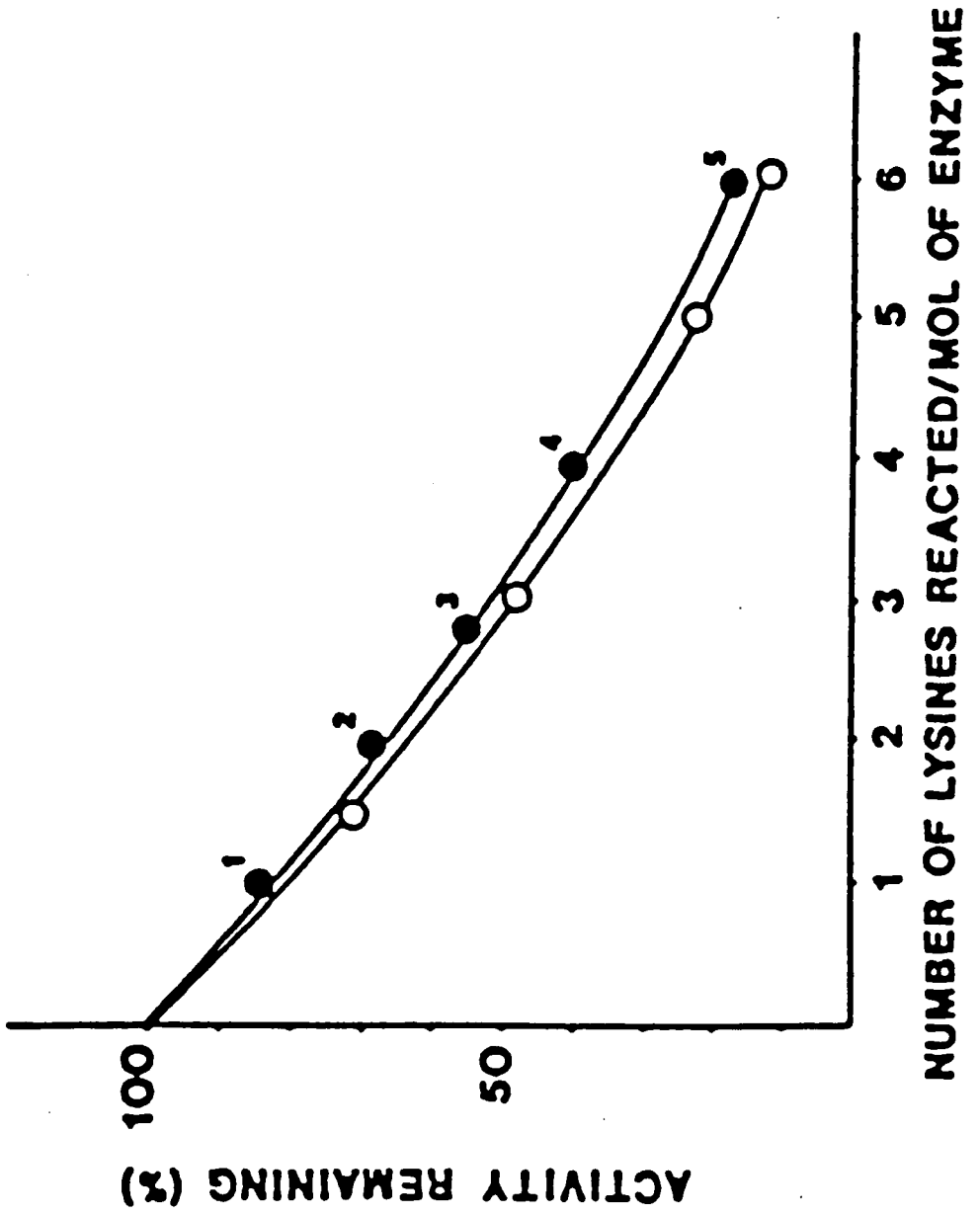

ACTIVITY REMAINING (%)
100
50
1
2
3
4
5
6
NUMBER OF LYSINES REACTED/MOL OF ENZYME

Figure 2.2

Figure 2.3

Absorption spectra of 4-aminobutyrate aminotransferase (1), 4-aminobutyrate aminotransferase reacted with 15 fold molar excess of o-phthalaldehyde (2), apoenzyme reacted with o-phthalaldehyde (3) and polyarginine reacted with o-phthalaldehyde (4).

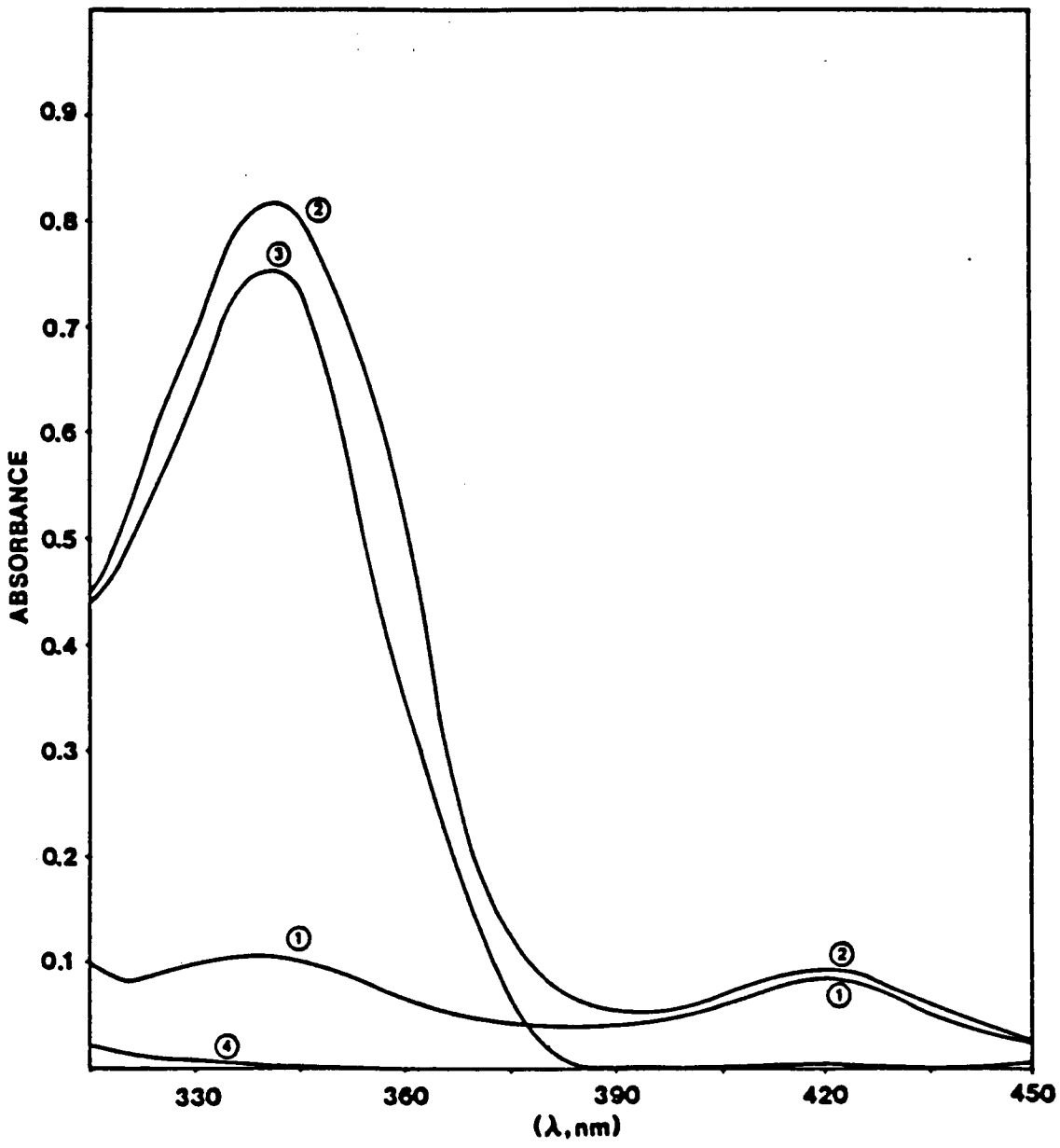

ABSORBANCE
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
330
360
390
420
450
(λ,nm)
1
2
3
4

Figure 2.3

Comparison of amino acid compositions between native and o-phthalaldehyde inactivated enzyme reveals differences only in the level of lysine; a sample of native enzyme contains 52 mol of lysine/mol of dimer, whereas a sample of enzyme 90% inactivated contains 46 mol of lysine/mol of dimer (Table 2.1).

III. DISCUSSION

The inactivation of 4-aminobutyrate aminotransferase by o-phthalaldehyde is a consequence of lysyl residues modification. Not only is o-phthalaldehyde specific for lysyl residues of the protein, but the reaction is restricted to a fraction of the total lysyl residues content. The enzyme contains 52 lysyl residues/dimer; of these only 6 are modified by o-phthalaldehyde leading to irreversible loss of catalytic activity.

There are two indications that the lysine residues, whose chemical modification caused inactivation, must lie close to the pyridoxal-5-P binding site. The first of this is of course, the sensitivity of 4-aminobutyrate aminotransferase toward o-phthalaldehyde. The second is the remarkable protection afforded by the substrate 2-oxoglutarate against the inactivating effect of o-phthalaldehyde. This is the most powerful evidence consistent with the concept that lysyl residues are involved in direct interaction with the carboxyl groups of the substrate.

The possibility that the modified lysyl residues are involved in binding of the cofactor pyridoxal-5-P via Schiff's base formation was also considered. The following experimental evidence strongly suggests that the latter possibility is not tenable.

TABLE 2.1

AMINO ACID COMPOSITION OF 4-AMINOBUTYRATE AMINOTRANSFERASE[a]
(residues/100,000 M.W.)

Amino Acid	Native	Modified[b]
Lysine	52	46
Histidine	20	19
Arginine	37	36
Aspartic Acid	80	81
Threonine	33	33
Serine	61	61
Glutamic Acid	79	78
Proline	47	47
Glycine	96	97
Alanine	76	75
Cysteine	6	6
Valine	42	42
Methionine	15	17
Isoleucine	39	41
Leucine	72	72
Tyrosine	17	17
Phenylalanine	43	43

a: The values are nearest integers based on the average of two independent determinations.
The amino acid composition was determined with an error of 4%.

b: Sample inactivated by o-PA mixing ratio (15:1).

a) The inactivation of 4-aminobutyrate aminotransferase by o-phthalaldehyde does not release the cofactor pyridoxal-5-P;

b) The inactivation profile of the apoenzyme reacted with o-phthalaldehyde is similar to that of the native enzyme; and

c) The addition of excess pyridoxal-5-P failed to protect the enzyme against inactivation by o-phthalaldehyde.

CHAPTER III

4-AMINOBUTYRATE AMINOTRANSFERASE, REACTION OF BIS-PLP WITH LYSYL RESIDUES CONNECTED WITH CATALYTIC ACTIVITY

Although the mechanism of action of 4-aminobutyrate aminotransferase is described by a ping-pong mechanism for bisubstrate enzymes (8,47), recent studies have shown that the holoenzyme possesses two non-equivalent catalytic binding sites differing in their affinities for the cofactor molecules (25). The catalytic behavior of 4-aminobutyrate aminotransferase is very unusual in the sense that the k_{cat} and K_m values of the enzyme containing 1 mol of pyridoxal-5-P/dimer are identical with those of the holoenzyme containing two mol of pyridoxal-5-P/dimer (25). This unusual behavior might be due to subunits which interact strongly in a negatively cooperative manner during the catalytic cycle.

Studies on amino acid residues which are related to catalytic function of 4-aminobutyrate aminotransferase have been reported in our laboratory (23,50). There is good evidence supporting the concept that conformational changes induced in one subunit by chemical modification are transmitted to the adjacent subunit with concommitant loss of catalytic activity. Thus, the blocking of one cysteinyl residue/dimer leads to complete loss of enzymatic activity, though the reactive cysteinyl group is not involved in the binding of either the substrate or the cofactor (23).

It is the purpose of the present work to gain more information about the amino acid residues involved in the binding of the substrates at the catalytic site. bis-PLP, an analogue of the cofactor pyridoxal-5-P, is used to probe the reactivity of lysyl residues connected with catalytic activity.

I. EXPERIMENTAL

Purification of the Enzymes

The purification of 4-aminobutyrate aminotransferase was performed by a method developed in our laboratory (25). The purification steps were conducted at 4°C. Succinic semialdehyde dehydrogenase was purified from pig brain by a method already described (42). The pyridoxal-5-P content of the purified aminotransferase was determined by the method of Wada and Snell (41). Protein concentration was determined by the colorimetric method of Lowry et al. (40). Apoenzyme was prepared by a method already described (25).

Enzymatic Assay

A coupled assay system consisting of two purified enzymes, i.e., 4-aminobutyrate aminotransferase and succinic semialdehyde dehydrogenase was used to monitor the catalytic conversion of 4-aminobutyrate into succinic semialdehyde.

Polyacrylamide Gel Electrophoresis

The enzyme preparations were examined by polyacrylamide gel electrophoresis according to the original procedure of Davis (43). Sodium dodecyl sulfate electrophoresis on polyacrylamide gels containing 7.5% polyacrylamide, 1% 2-mercaptoethanol and 0.1% sodium dodecyl sulfate was performed according to the method of Weber and Osborn (44). Protein bands were detected by staining with coomassie blue dye.

Spectroscopy

Spectrophotometeric measurements were carried out in either a Cary Model 15 or an Aminco DW-2 Double Beam Spectrophotometer. Fluorescence

spectra were recorded in a precision spectrofluorimeter equipped with two Bausch and Lomb Monochromators. The slits of the monochromators were set to give a band width of 3 nm.

Synthesis of bis-PLP

P^1,P^2-bis(5'-pyridoxal)diphosphate was synthesized following the procedure of Shimomura and Fukui (49). The R_f value for bis-PLP on TLC (Kieselgel, Merck) is 0.48 using the solvent system n-butanol-pyridine-water (60:40:30, v/v/v). The UV spectra showed λ_{max} 294 nm and λ_{max} 335 nm in 0.1 N HCl, and λ_{max} 391 nm (ε = 9,400) in 0.1 M phosphate buffer (pH 7.0).

bis-PNP was prepared by reduction with $NaBH_4$. The UV spectrum shows an absorption band centered at 324 nm (ε= 15,000) at pH 7.0.

bis-P-pyridoxyl-lysine was prepared by reacting bis-PLP with polylysine, followed by reduction with $NaBH_4$ and digestion with trypsin. The UV spectrum shows an absorption band centered at 325 nm (ε = 18,400) at pH 7.0. The spectroscopic properties are identical with those of P-pyridoxyl-lysine.

Reaction with bis-PLP

The reaction of the aminotransferase with bis-PLP was monitored at 450 nm under pseudo-first order conditions. The observed rate constant was obtained from a plot of log (A_∞ - A_t) versus time according to the following equation:

$$(A_\infty - A_t) = (A_\infty - A_0)\ e^{-k_{obs}\,.t} \qquad [1]$$

Where A_∞, A_t and A_0 are the absorbance values at time ∞, t and zero, respectively. The degree of labelling of the protein was estimated spectrophotometrically using an exctinction coefficient of $2.7 \times 10^3\ M^{-1}\ cm^{-1}$ at 450 nm. It is the exctinction coefficient obtained when bis-PLP (10^{-4}M) is allowed to react with β-alanine (10^{-2}M) at pH 7.0.

II. RESULTS

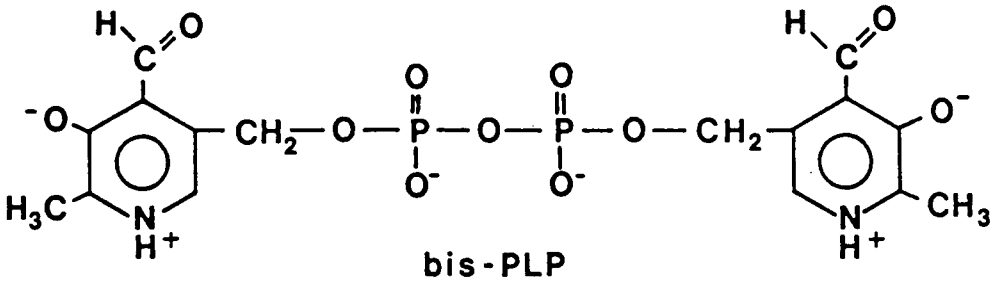
Inactivation of 4-Aminobutyrate Aminotransferase

4-Aminobutyrate aminotransferase reacts with bis-PLP (Fig. 3.1), which is a specific reagent for ε-amino groups of lysyl residues in proteins (49). The effect of addition of bis-PLP on the catalytic activity of the aminotransferase was investigated by preincubating the enzyme with increasing concentrations of bis-PLP at 25°C in 0.1 M phosphate buffer (pH 7.0). As shown in Fig. 3.2, inactivation of the enzyme was attained when the protein was preincubated with 20 fold molar excess of bis-PLP at pH 7.0 for 60 minutes. The reaction is irreversible; the catalytic activity of the aminotransferase can not be restored by addition of excess pyridoxal-5-P followed by incubation for 1 hour at 25°C. Complete protection against the inhibitory effect of the bifunctional reagent was observed by preincubating the enzyme with 10 mM α -ketoglutarate prior to the addition of bis-PLP. The time course of inactivation of the enzyme in the presence and absence of α -ketoglutarate is given in Fig. 3.3. It should be noted that pyridoxal-5-P (0.1 mM) failed to affect the rate and extent of inactivation of the aminotransferase as indicated by the results of Fig. 3.3. Since pyridoxal-5-P neither inhibits nor protects the enzyme against the reaction with bis-PLP, it seems likely that immobilization of bulky bis-PLP by positive charges on the enzyme facilitates the reaction of its carbonyl groups with lysyl residues.

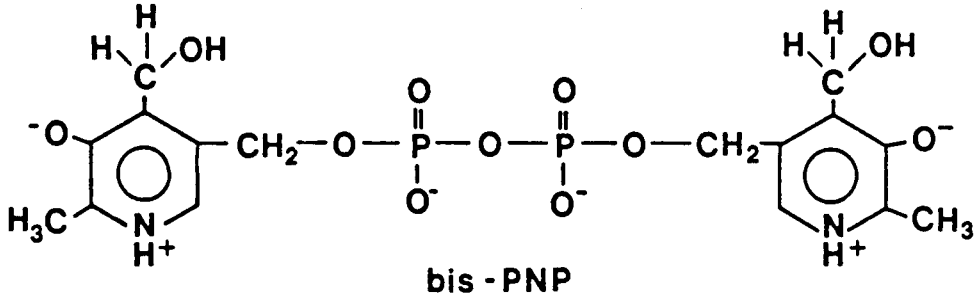
The time course of inactivation of 4-aminobutyrate aminotransferase is significantly affected by variations in the pH of the reaction mixtures. Fig. 3.4 shows the kinetic results obtained when the enzyme (20 μ M) was allowed

Figure 3.1

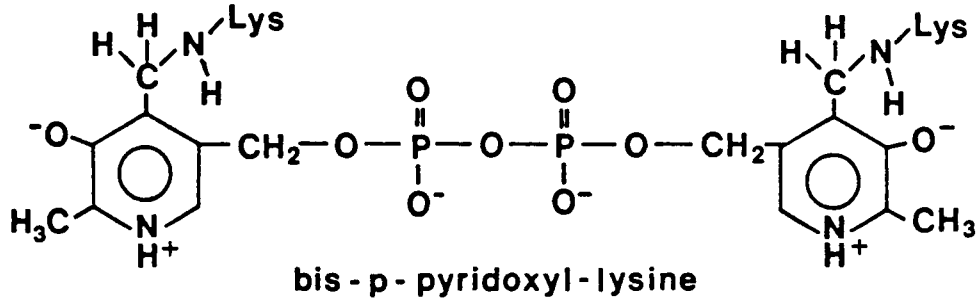
Structure of bis-PLP.



bis-PLP



bis-PNP



bis-p-pyridoxyl-lysine

Figure 3.1

Figure 3.2.

Inhibition of 4-aminobutyrate aminotransferase by bis-PLP at pH 7.0 in 0.1M phosphate buffer. The enzyme (14 μ M) was preincubated with varying concentrations of bis-PLP at 25°C for 1 hour in the presence of 10 mM α -keotglutarate (curve 1) and in the absence of α -ketoglutarate (curve 2). Aliquots withdrawn from the incubation mixtures were tested for enzymatic activity. A sample of apoenzyme (14 μM) was preincubated with varying concentrations of bis-PLP at 25°C for 1 hour. The samples were then reacted with pyridoxal-5-P (0.1 mM) for 1 hour at 25°C prior to the enzymatic assays (curve 3).

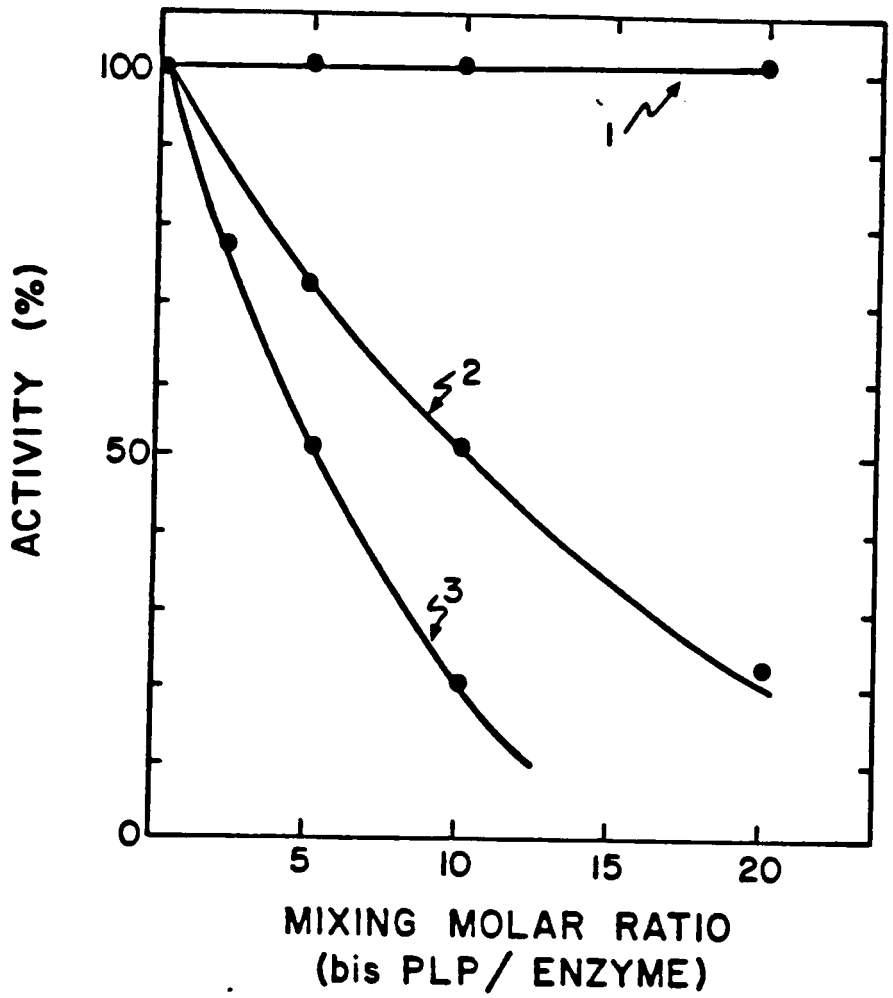

100
50
0
ACTIVITY (%)
1
2
3
5
10
15
20
MIXING MOLAR RATIO
(bis PLP/ ENZYME)

Figure 3.2.

Figure 3.3

4-Aminobutyrate aminotransferase (20 μ M) was incubated with 10 fold molar excess of bis-PLP (curve 2) and with 20 fold molar excess of bis-PLP (curve 3) in the absence of α-ketoglutarate. Results obtained when 10 mM α-ketoglutarate were added to 20 μM of the enzyme prior to the addition of 10 fold (o) and 20 fold excess of bis-PLP (●) are given in curve 1. The decay of enzymatic activity of a sample of enzyme (20 μ M) preincubated with 0.1 mM pyridoxal-5-P prior to the addition of 20 fold molar excess of bis-PLP is given in curve 4 (o).

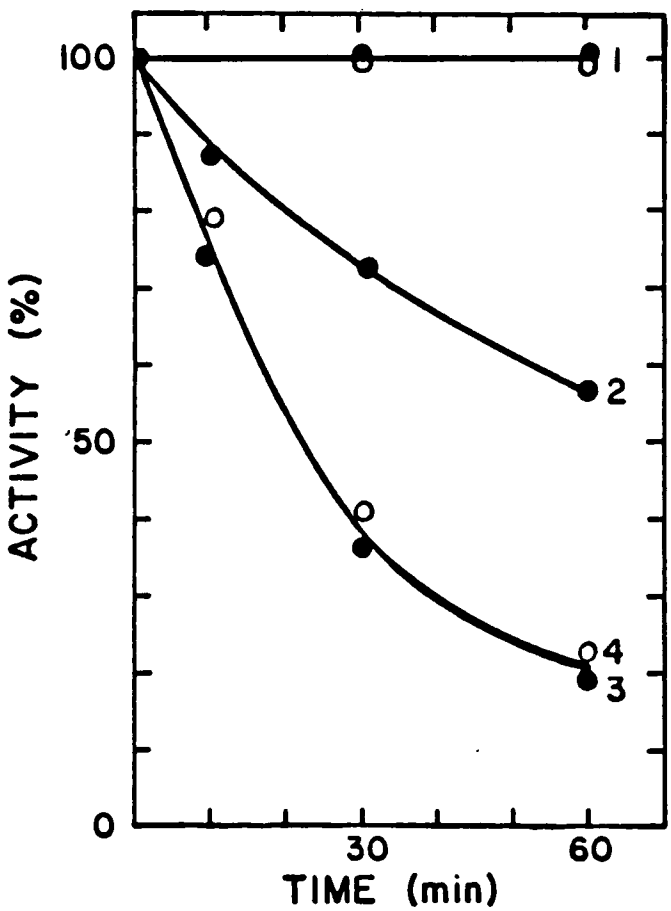


Figure 3.3

Figure 3.4

Rate of inactivaiton of 4-aminobutyrate aminotransferase at different pH values. Samples of enzyme (20 μ M) were incubated with 10 fold excess of bis-PLP at pH 6.6 (1), 7.0 (2), 7.6(3), 8.1(4) and 8.6(5) at 25°C. Aliquots withdrawn from the incubation mixtures were tested for enzymatic activity. Plot of log activity versus incubation time.

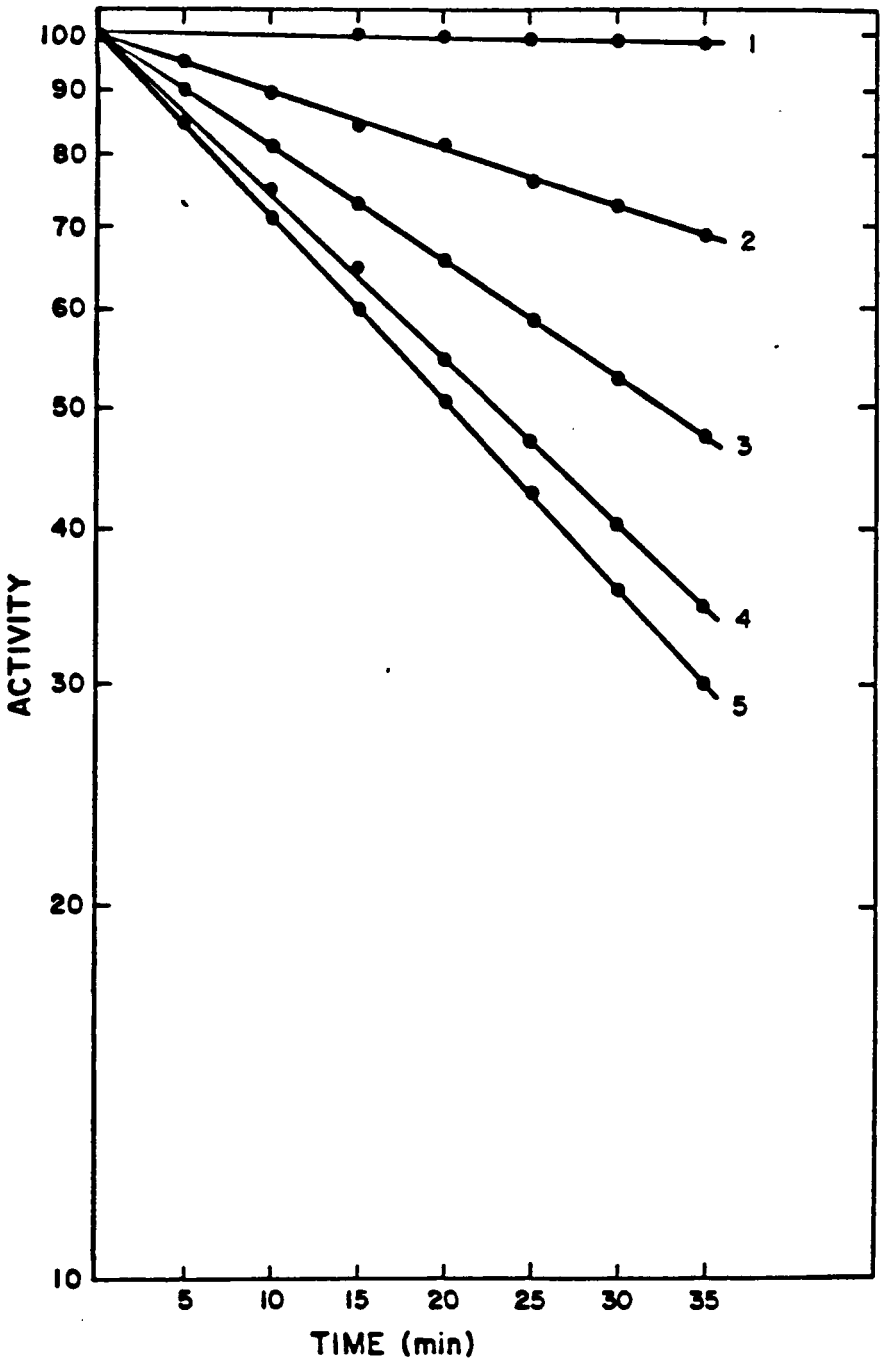

100
90
80
70
60
50
40
30
20
10
ACTIVITY
5
10
15
20
25
30
35
TIME (min)
1
2
3
4
5

Figure 3.4

to react with 10 fold molar excess of bis-PLP at various pH values at constant temperature (25°C).

If the reaction of the lysyl groups of the protein with bis-PLP can be described by the simple reaction scheme:

$$\text{Bis-PLP} + \text{P-NH}_2 \xrightarrow{k_o} \text{bis-PLP-P}$$

$$\text{K} \Big\| \uparrow$$

$$\text{P-NH}_3^+$$

Then, the observed rate constant of inactivation (k_{obs}) is related to the dissociation constant (K) and the proton concentration (H^+) by equation [2]

$$k_{obs} = \frac{k_o (PLP)_2}{1 + (H^+)/K} \qquad [2]$$

where k_o is the second order rate constant and $(PLP)_2$, the initial concentration of bis-PLP. The values of k_{obs}, obtained from the results included in Fig. 3.4, are plotted as a function of pH in Fig. 3.5. The experimental values fit well a theoretical curve calculated for $K = 10^{-7.3}$ M and $k_o = 1.7 \times 10^2$ M^{-1} min^{-1}.

Hence, the attacking reagent bis-PLP blocks lysyl residues characterized by pK values lower than those of the normal ε-amino group of lysyl residues in proteins (pK = 9.4).

Figure 3.5

Plot of $k_{obs}(min^{-1})$ versus pH. The observed rate constant of inactivation (k_{obs}) (o) was determined from the results included in Fig. 3. The theoretical values (•) were calculated for pK = 7.3 and k_o = 1.7 X 10^2 $M^{-1}min^{-1}$ (equation 2).

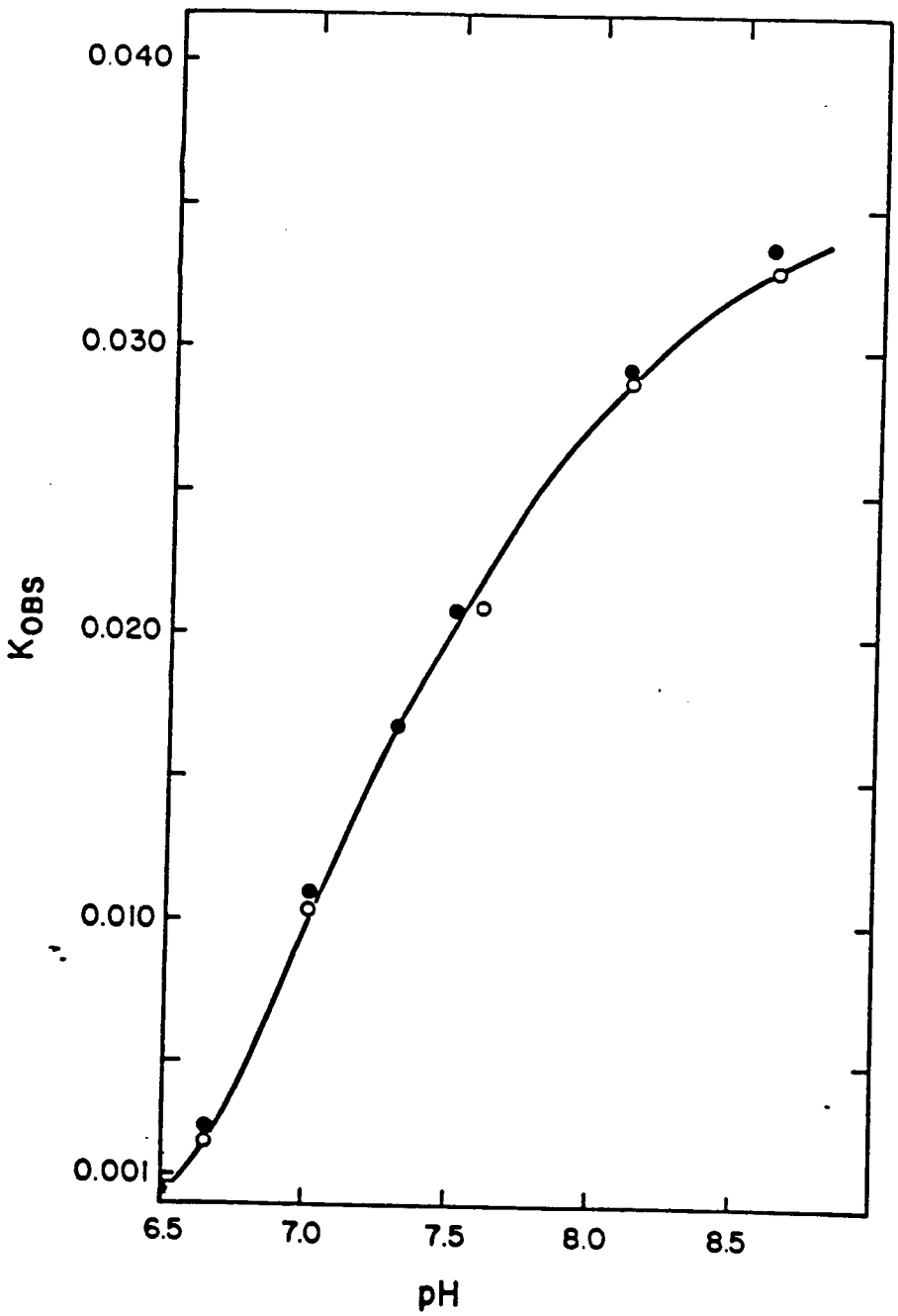


Figure 3.5

Reaction of Lysyl Residues of the Enzyme

To ascertain whether the loss of catalytic activity produced by reaction with bis-PLP could be related to the modification of few lysyl residues, absorption spectra were taken for samples of enzyme reacted with bis-PLP at pH 7.0 in 0.1 M phosphate buffer.

bis-PLP exhibits an intense absorption band centered at 391 nm due to the presence of two aldehyde groups per molecule (49). The reaction of bis-PLP with ε-amino groups of lysyl residues leading to the formation of Schiff's base adducts is accompanied by characteristic changes in the absorption spectrum of the modifier; the absorbance at 391 nm decreases with a concomitant increase in absorbance at wavelengths longer than 400 nm (49). When the absorbance of samples of enzyme reacted with bis-PLP were monitored at 450 nm, the kinetic profiles included in Fig. 3.6 were obtained. The sample of apoenzyme, incubated with 20 fold molar excess of reagent for 45 minutes at 25°C, has lost the ability to regain any enzymatic activity upon subsequent addition of pyridoxal-5-P. The increase in absorbance at 450 nm after 45 minutes incubation with bis-PLP corresponded to the reaction of 2 bis-PLP molecules/dimer (Table 3.1).

In marked contrast to the apoenzyme, the native enzyme has lost 95% of its original enzymatic activity as result of the reaction of only 1.2 bis-PLP molecules/dimer (Fig. 3.6). Thus, the presence of one molecule of cofactor/dimer has a dramatic effect on the rate and extent of reaction with bis-PLP (Table 3.1). The determination of the degree of labelling of apoenzyme and holoenzyme was based on the assumption that the extinction coefficient of bound bis-PLP is identical with that of the Schiff's base formed between bis-PLP and β-alanine ($\varepsilon_{450} = 2.7 \times 10^3\ M^{-1}\ cm^{-1}$). However, variations in

Figure 3.6

Reaction of the apoenzyme (10 μM) (Curve 1) and holoenzyme (10 μM) (Curve 2) with bis-PLP (200 μM) at 25°C (pH 7.0). The increase in absorbance at 450 nm is plotted as a function of the time of incubation. The results were analyzed by equation (1) and the k_{obs} values for the apoenzyme and holoenzyme are 0.05 and 0.03 min^{-1}, respectively.

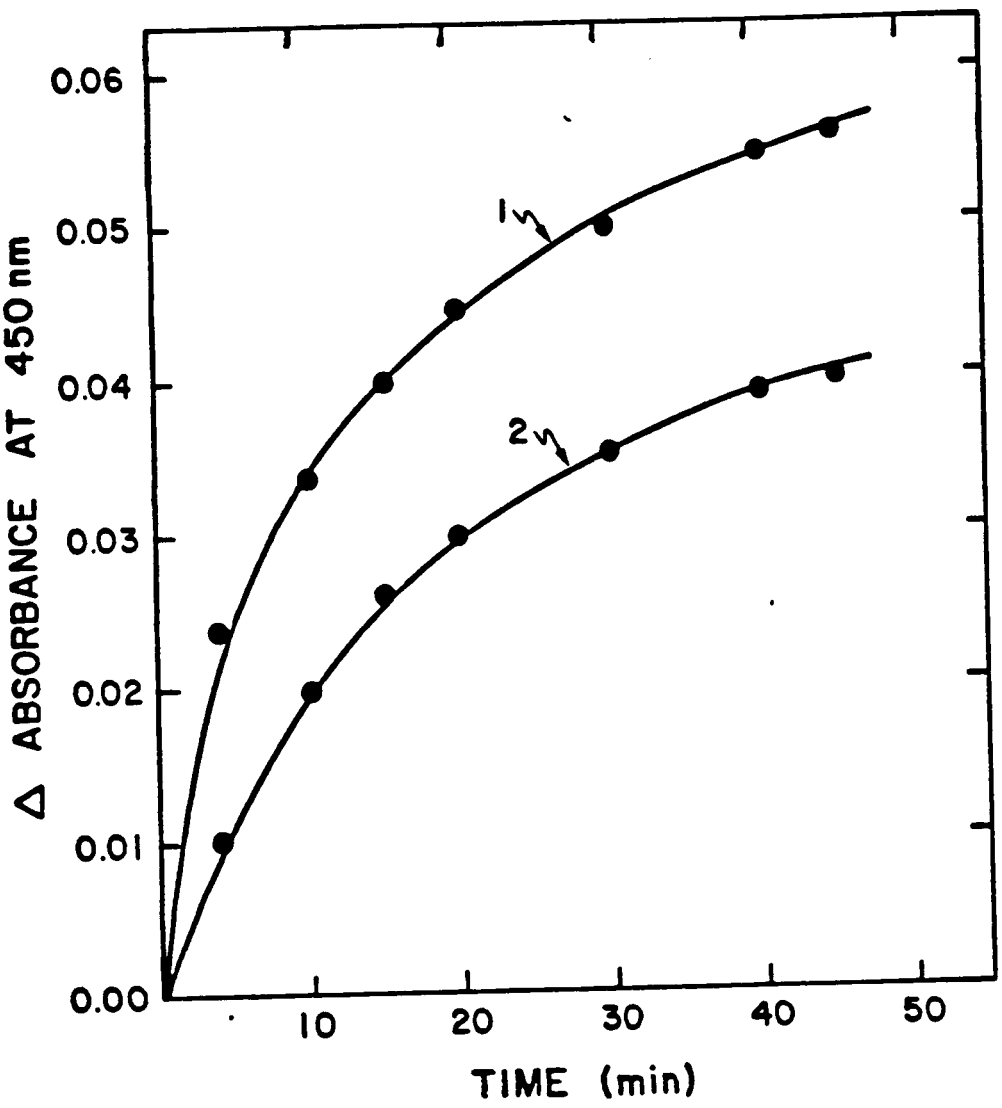

0.06
0.05
0.04
0.03
0.02
0.01
0.00
Δ ABSORBANCE AT 450 nm
1
2
10
20
30
40
50
TIME (min)

Figure 3.6

TABLE 3.1

REACTION OF 4-AMINOBUTYRATE AMINOTRANSFERASE (10 μM) WITH bis-PLP (200 μM) AT pH 7.0, 25°C

Enzyme Sample	Activity[a]	k_{obs}[b] (min^{-1})	bis-PLP/Dimer[c]	P-pyridoxyl /Dimer[d]	P-pyridoxine /Dimer[e]
Holoenyzme + bis-PLP	5%	0.03	1.2	2.1	2.2
Apoenzyme + bis-PLP	0	0.05	2	3.9	3.7

a: Enzymatic activity determined after 45 min. reaction with bis-PLP.

b: Determined by measuring the increase in absorbance at 450 nm.

c: Determined after 45 min. incubation with bis-PLP.

d: Determined after reduction with $NaBH_4$ and dialysis, using ϵ = 9,200 at 325 nm.

e: Determined after reduction with $NaBH_4$ and dialysis, using ϵ = 7,500 at 325 nm.

the molar extinction coefficient were observed when bis-PLP was allowed to react with proteins. In the case of apoaspartate aminotransferase, an extinction coefficient of 3.3×10^3 M^{-1} cm^{-1} was determined at 450 nm after the bifunctional reagent was incorporated into the protein. For this reason, i.e. uncertainty in the value of the molar extinction coefficient of bis-PLP bound to 4-aminobutyrate aminotransferase, we decided to determine the extent of chemical modification of the enzyme by resorting to other spectroscopic methods.

Spectroscopy of the labelled enzyme

When the absorption spectrum of the sample of enzyme reacted with bis-PLP, reduced with $NaBH_4$, and dialyzed overnight at 4°C, was recorded in the spectral range 300-360 nm, it was observed that an intense absorption band corresponding to either P-pyridoxine or P-pyridoxal chromophores was detectable at 325 nm (Fig. 3.7). The emission spectrum of the labelled enzyme also displayed an intense fluorescence band centered at around 395 nm.

Table 3.2 includes the spectroscopic properties of the labelled enzyme, together with the properties of the model compounds, pyridoxine-5-P, bis-pyridoxine-5-P and P-pyridoxyl-lysine. Like pyridoxine-5-P, bis-pyridoxine-5-P interacts with borate ions to form a stable adduct which absorbs maximally at 290 nm (Fig. 3.7). The equilibrium dissociation constant for the adduct is 1 mM at pH 7.0.

P-Pyridoxyl-lysine does not form a complex with borate, and as indicated by the results of Table 3.1, the absorption band at 325 nm remains essentially invariant upon addition of borate. Hence, the degree of labelling of the enzyme reacted with bis-PLP and reduced with $NaBH_4$ can be determined by recording

Figure 3.7

Absorption spectra of bis-pyridoxine-5-P in 0.1 M borate (pH 7.0) (1) and in 0.1 M phosphate (pH 7.0) (2). Absorption spectra of the enzyme (8.3 μ M) after reaction with bis-PLP and reduction with $NaBH_4$ at pH 7.0 in 0.1 M phosphate (3) and 0.1 M borate (4). The absorption spectrum of the native enzyme (8.3 μ M) reduced with $NaBH_4$ and dialyzed against 0.1 M borate (pH 7.0) is included in the figure (curve 5). The degree of labelling with P-pyridoxyl was determined by using the difference in absorbance at 325 nm of curves 4 and 5. The degree of labelling with P-pyridoxine was determined by using the difference in absorbance at 325 nm of curves 3 and 4.

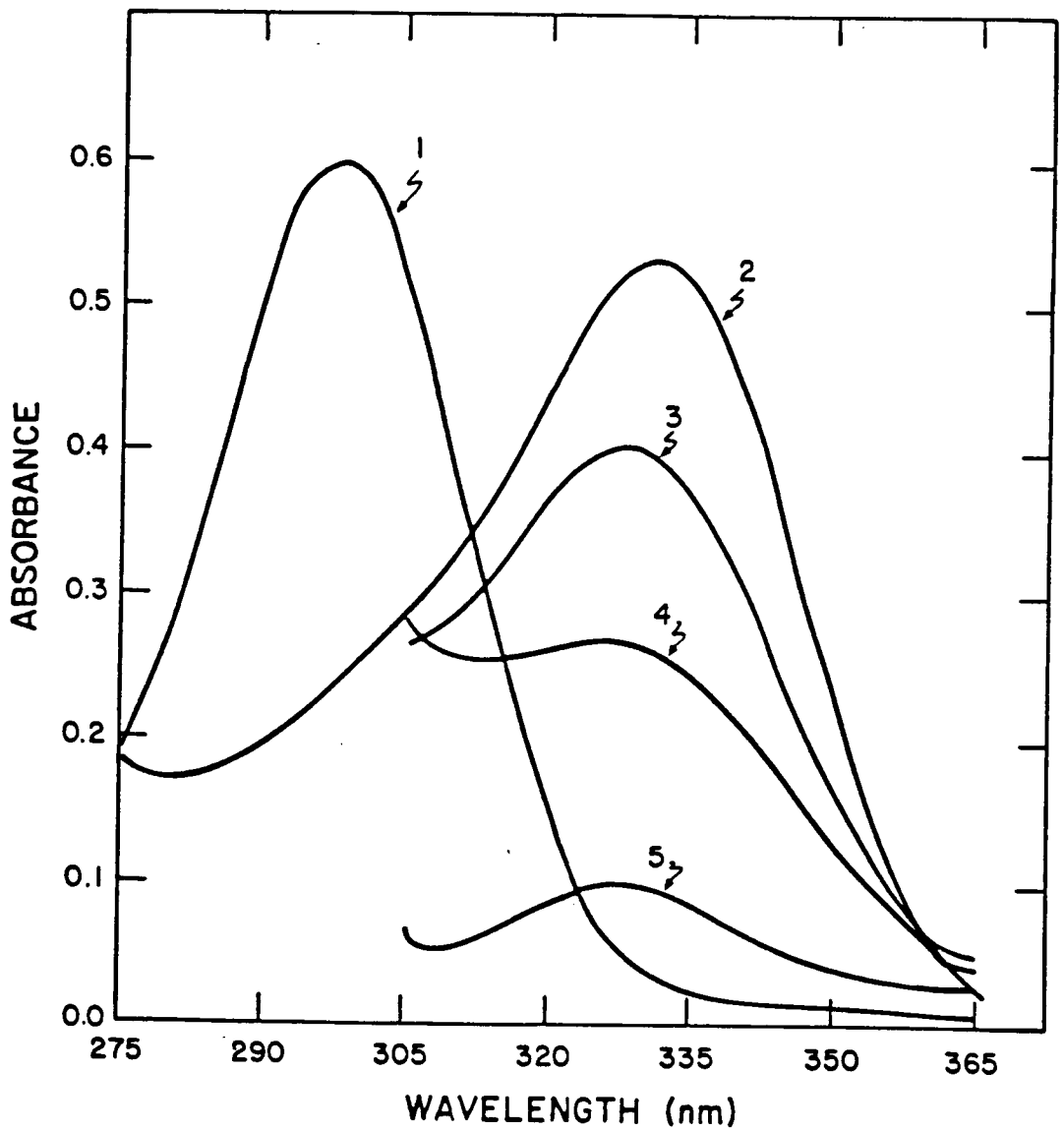

ABSORBANCE
0.0
0.1
0.2
0.3
0.4
0.5
0.6
275
290
305
320
335
350
365
WAVELENGTH (nm)
1
2
3
4
5

Figure 3.7

TABLE 3.2

SPECTROSCOPIC PROPERTIES of bis-PYRIDOXINE-5-P and bis-P-PYRIDOXYL-LYSINE

Sample	pH	Absorption	Fluorescence
		(λ,nm)	(λ,nm)
pyridoxine-5-P	7.0	324	390
pyridoxine-5-P	2.0	292	390
pyridoxine-5-P	7.0, 0.1 M borate	290	335
bis-pyridoxine-5-P	7.0	324	390
bis-pyridoxine-5-P	2.0	292	390
bis-pyridoxine-5-P	7.0, 0.1 M borate	290	335
bis-P-pyridoxyl-lysine	7.0	325	392
bis-P-pyridoxyl-lysine	2.0	292	395
bis-P-pyridoxyl-lysine	7.0, 0.1 M borate	325	392

its absorption spectra in the presence and absence of borate ions. If both carbonyl groups of bis-PLP have reacted with lysyl residues of the protein, then the intensity of the absorption band of the reduced enzyme at 325 nm should remain unperturbed by the presence of borate ions. If, on the other hand, a fraction of the carbonyl groups of the bifunctional reagent has not reacted with the protein, then the absorption band at 325 nm should be perturbed by addition of borate. Fig. 3.7 shows the absorption spectra of a sample of enzyme inactivated by bis-PLP and reduced with $NaBH_4$ The intensity of the absorption band at 325 nm in the presence of 0.1 M borate was used to calculate the number of P-pyridoxyl residues, whereas the difference in absorbance at 325 nm in the absence and presence of borate, was used to determine the number of P-pyridoxine residues. The results of these measurements yield 2.1 P-pyridoxyl residues and 2.2 P-pyridoxine/per enzyme dimer (Table 3.1).

The ratio of P-pyridoxyl to P-pyridoxine chromophores is within experimental error equal to one, indicating that the bifunctional reagent, whose expanded structure covers a distance of the order of 17Å (49), has failed to crosslink lysyl residues located on different subunits. Further support for this contention was derived from electrophoresis experiments conducted on sodium dodecyl sulfate polyacrylamide gels. The polyacrylamide gel patterns of samples labelled with bis-PLP and reduced with $NaBH_4$, exhibited a major component of 50,000 molecular weight (Fig. 3.8). Its apparent molecular weight is close to that expected if dissociation of the enzyme (100,000 mw) into identical monomers has taken place after denaturation with sodium dodecyl sulfate.

Figure 3.8

Sodium dodecyl sulfate polyacrylamide gel electrophoresis pattern of samples of 4-aminobutyrate aminotransferase reacted with bis-PLP and reduced with $NaBH_4$. Results of four independent experiments. The molecular weights of standards are myosin, 200,000; phosphorylase, 93,000; bovine serum albumin, 69,000; and ovalbumin, 43,000.

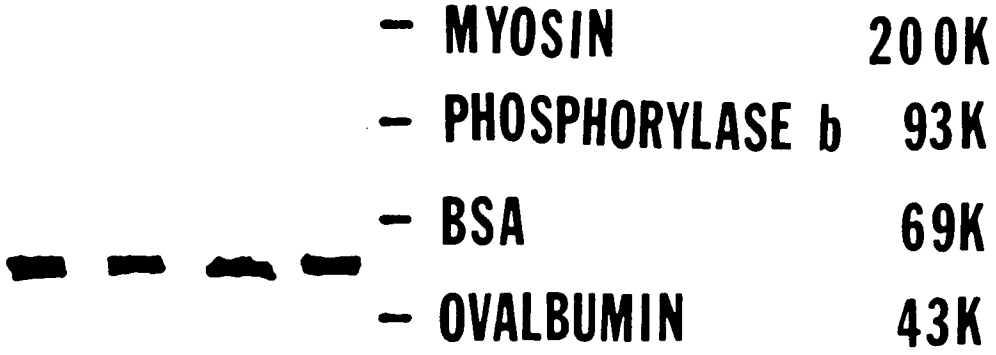


Figure 3.8

III. DISCUSSION

Several lines of experimental evidence presented in this work indicate that the bifunctional reagent bis-PLP is a suitable probe for studying the reactivity of lysyl residues in 4-aminobutyrate aminotransferase. The spectroscopic properties of P-pyridoxyl and P-pyridoxine chromophores, produced after reduction of the protein treated with bis-PLP, can be used to determine the degree of labelling of the modified enzyme as well as the fraction of P-pyridoxyl residues involved in crosslinkage.

The modification of few lysyl residues (2.1 lysyl residues/dimer) in the aminotransferase brings about an irreversible loss of catalytic activity. Not only is bis-PLP specific for lysyl residues in proteins, but the reaction is restricted to a small fraction of the total lysyl content. The enzyme isolated from pig brain contains 52 lysyl residues/dimer (50) of these only 2.1 lysyl residues/dimer are readily accessible to chemical modification at neutral pH.

The predominant species obtained after sodium dodecyl sulfate polyacrylamide gel electrophoresis corresponded to the molecular weight of the monomer of 4-aminobutyrate aminotransferase, indicating that P-pyridoxyl residues are not involved in the crosslinking of lysyl residues located on different subunits. There are several indications that those lysyl residues, whose chemical modification caused enzyme inactivation, must lie close to the cofactor binding site. The first is of course the sensitivity of 4-aminobutyrate aminotransferase to lysyl reagents. The reaction of a very small fraction of the total lysyl content with either bis-PLP or o-phthalaldehyde (50) leads to irreversible loss of catalytic activity. The second is the protection afforded by α-ketoglutarate

against the inactivating effect of the lysyl reagents. This is the most powerful evidence consistent with the concept that lysyl residues are involved in direct interaction with the carboxyl groups of the substrate. The protection afforded by α -ketoglutarate may be discussed in reference to a model which assumes that binding of α-ketoglutarate is the major factor contributing to stabilization of the catalytic site. Alternatively, there is the possibility that α -ketoglutarate bound to the cofactor binding site prevents displacement of pyridoxal-5-P by the attacking bifunctional reagent. While this explanation seems attractive, it may be disputed on the basis of the lack of protection afforded by exogeneous pyridoxal-5-P at concentrations considerably higher than the dissociation constant of the cofactor (K_d = 1 nM). The differential spectra of samples of apoenzyme and holoenzyme inactivated with bis-PLP and dialyzed against buffer (pH 7.0) shows the presence of an absorption band centered at 415 nm, which can be attributed to the presence of covalently bound pyridoxal-5-P (data not shown). Similar results were reported (50) for the enzyme inactivated with o-phthalaldehyde. Hence, the irreversible inactivation of 4-aminobutyrate aminotransferase upon reaction with bis-PLP cannot be ascribed to dissociation of the cofactor from the catalytic site.

Although the precise functional role of lysyl residues in catalysis remains to be elucidated, these results indicate that chemical modification of two lysyl residues is needed for inactivation of each subunits.

CHAPTER IV

CATALYTIC AND STRUCTURAL PROPERTIES OF TRYPSIN TREATED 4-AMINOBUTYRATE AMINOTRANSFERASE

The existence of multiple forms of 4-aminobutyrate aminotransferase isolated from brain tissues has been reported by two independent groups (6,13). More recently, it was demonstrated that the endopeptidase cathepsin-T generates multiple forms of tyrosine aminotransferase (60).

Previous work in our laboratory has shown that the dissociation constant for the first catalytic binding site of 4-aminobutyrate aminotransferase is 1 nM, whereas the second catalytic site is characterized by a dissociation constant of 3 μM (25). This unusual characteristics might reflect negative cooperative interaction between subunits.

A cooperative interaction between subunits implies communication within the dimeric structure and propagation of conformational changes between the adjacent monomers. Attempts to dissociate the enzyme and to study enzymatically active monomers have been unsuccesful.

In order to study the stability of the dimeric structure in solution, it was attempted to investigate whether selective cleavage of the enzyme by proteolysis would perturb the area of contact between the subunits and abolish negative cooperativity.

It is the purpose of this work to report the effect of trypsin digestion on the structural and catalytic properties of 4-aminobutyrate aminotransferase.

I. EXPERIMENTAL

Purification of Enzymes

4-aminobutyrate aminotransferase from pig brain was purified according to a procedure previously described (25). This preparation has a specific activity of 20 units/mg at 25°C and it migrates as a single protein band on polyacrylamide gel electrophoresis.

Protein concentration was determiend by the colorimetric method of Lowry et al. (40). The pyridoxal-5-P content of the purified aminotransferase was determined by the method of Wada and Snell (41). The enzyme succinic semialdehyde dehydrogenase from pig brain was purified by a method already described (42).

Enzymatic Assays

A coupled assay system consisting of two purified enzymes, i.e. 4-aminobutyrate aminotransferase and succinic semialdehyde dehydrogenase, was used to study the catalytic conversion of 4-aminobutyrate to succinic semialdehyde.

Titration of Thiol Groups

The number of reactive thiol groups was determined by reaction with 5,5', dithiobis-(2-nitrobenzoic acid), DTNB, using the procedure of Ellman (53). A molar exctinction coefficient of 13,600 $M^{-1}cm^{-1}$ at 412 nm was used for the determination of the concentration of the anion 2-nitro-5-mercaptobenzoate (MNB).

Labeling of the Enzyme

4-aminobutyrate aminotransferase at a concentration of 5 mg/ml was allowed to react with 1mM IAF (6-iodoacetamide fluorescein) in 0.1 M potassium phosphate (pH 7) at 4°C. The reaction was allowed to proceed for 5 hours at 4°C. Excess of free reagent was removed by gel filtration through Sephadex G-25 equilibrated with 0.1 M potassium phosphate (pH 7) containing 0.1 mM 2-mercaptoethanol. The degree of labelling of the enzyme was determined spectrophotometrically using an exctinction coefficient of $3.4 \times 10^4 M^{-1} cm^{-1}$ at 490 nm. The incorporation of 0.92 mol of dye/ mol of enzyme does not affect the catalytic activity.

Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis was pereformed according to the original procedure of Davis (43). Discontinuous sodium dodecyl sulfate/polyacrylamide gel electrophoresis was carried out at 25°C as described by Lamelli (54). The gel (15% acrylamide) contained 0.1% sodium dodecyl sulfate.

Polyacrylamide gradient gel electrophoresis (4% - 30%) was performed at 10°C, using a voltage of 125 volts for 15 hours. Standards of known molecular weight, bovine serum albumin, catalase and ceruloplasmin were used in the plots of 1/XL versus $(MW)^{1/3}$ following the procedure of Manwell (55).

Protein bands were detected by staining with Coomassie blue dye for 1 hour and subsequently destained overnight in a solution containing 10% methanol and 7% acetic acid in water.

Tryptic Digestion of 4-Aminobutyrate Aminotransferase

4-Aminobutyrate aminotransferase (5 mg/ml) was incubated with trypsin (0.025 mg/ml) in 50 mM ammonium bicarbonate (pH 7.6) at 37°C for 2 hours. At the end of the incubation, the mixture was filtered through a column of Sephadex G-75 (2 x 100 cm) equilibrated with 50 mM ammonium bicarbonate (pH 7.6) at 4°C.

The fractions eluted from the column were monitored by absorbance measurements at 280 nm. The protein eluted in the void volume of the column was dialyzed against 0.1 M phosphate buffer (pH 7) containing 0.1 mM 2-mercaptoethanol and used for further characterization experiments.

All the fractions eluted between the excluded and included volumes of the column were combined and lyophilized.

Amino Acid Analysis

Acid hydrolysis was carried out in 4N methanesulfonic acid (56) and in 6N HCl at 110°C in sealed evacuated tubes for 24 hours. The amino acid composition of the peptide mixture before and after acid hydrolysis was determined in a Jeol-6-AH autoanalyzer using the expansion scale of 0.15 absorbance as full scale.

Fingerprint Analysis

It was performed using the method of Schiltz and Reinbolt (57). The sample was applied to a microcrystalline cellulose plate. (Polygram-CEL 400) and electrophoresis in the first dimension was conducted at 600 volts using the solvent system pyridine/acetic acid/H_2O/acetone (2:4:79:15 v/v/v/v, pH 4.4). Chromatography in the second dimension was performed using the solvent

system N-butanol/Pyridine/acetic acid/H_2O (15:10:3:12). The thin-layer sheets were sprayed with fluorescamine (0.01% w/v) in acetone and post sprayed with 10% (v/v) triethylamine in dichloromethane.

Analytical Ultracentrifugation

Sedimentation velocity experiments were conducted on the Spinco model E analytical ultracentrifuge at constant temperature in the range of 8-10°C. For sedimentation velocity experiments, the ultracentrifuge was operated at 60,000 RPM and the sedimentation coefficients were corrected for the density and viscosity of water at 20°C. Samples of enzyme at concentrations varying between 3 and 10 μM in 0.1 M potassium phosphate, pH 7.0 were studied in the analytical ultracentrifuge equipped with a photoelectric scanner (58).

Spectroscopy

Absorption spectra were recorded on a Cary model 15 spectrophotometer. Fluorescence emission spectra were recorded on a spectrofluorimeter equipped with two Bausch and Lomb monochromators. The slits of the monochromators were set to give a band width of 3 nm.

Polarization of fluorescence measurements were performed on a SLM 4800 spectofluorimeter. The excitation was set at 440, and fluorescence polarized light emitted by the sample was passed through a Corning glass filter CS 3-69.

Fluorescence decay measurments were made using the monophoton technique with an Ortec Model 9200 Nanosecond spectrofluorimeter. A free running lamp operating in air at 1 atm pressure was the exciting light source. The lamp was pulsed at 10 kHz. Excitation was set at 440 nm and the emission was filtered through a Corning glass filter CS 3-69. Deconvolution of the

data was performed with a computer program based on the Phase-Plane method of Demans and Adamson (59).

Materials

Pig brains were obtained from East Tenn. Packing Co., Knoxville. Trypsin was purchased from Worthington Biochemical Company. Sephadex G-25, G-200, G-75, DEAE-Sephadex, CM-Sephadex and polyacrylamide gradient gels (PAA 4/30) from Pharmacia Fine Chemicals. Acrylamide, N-N'-methylenebisacrylamide, and standards for protein molecular weight from Bethesda Research Laboratories. 5.5'-Dithiobis (2-nitrobenzoic acid), DTNB, from Aldrich Chemical Co. and 6-iodoacetamido fluorescein (IAF) from Molecular Probes.

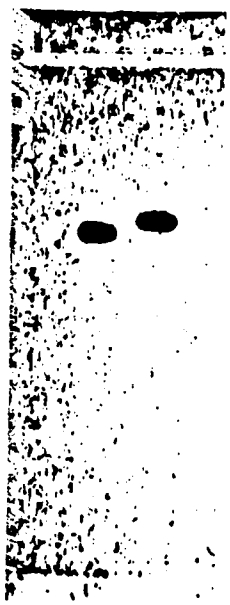
II. RESULTS

Digestion of 4-Aminobutyrate Aminotransferase

4-aminobutyrate aminotransferase (2 mg/ml) was incubated with trypsin (10 μ g/ml) in 50 mM ammonium bicarbonate (pH 7.6) at 37°C. After various incubation times, samples were removed for enzyme assay, and for polyacrylamide gel electrophoresis. The overall catalytic activity of 4-aminobutyrate aminotransferase after 2 hours incubation at 37°C was observed to be unaffected by trypsin addition, whereas the polyacrylamide gel electrophoresis patterns revealed the presence of mainly one band displaying different mobility than the native enzyme (Figure 4.1). If digestion with trypsin has taken place, the peptides produced are of small molecular weight and they migrate rapidly in the gel electrophoresis system used (7.5% acrylamide).

Figure 4.1

Effect of trypsin digestion on the catalytic activity of 4-aminobutyrate aminotransferase. The enzyme (2 mg/ml) was incubated with trypsin (10_{μ} g/ml) at pH 7 (●) pH 7.4 (0) and pH 7.6 (▽) at 37°C. Aliquots removed from the incubation mixtures were tested for enzymatic activity. Polyacrylamide (7.5%) Gel electrophoresis pattern of native and enzyme treated with trypsin. The sample was digested for 2 hours at 37°C.



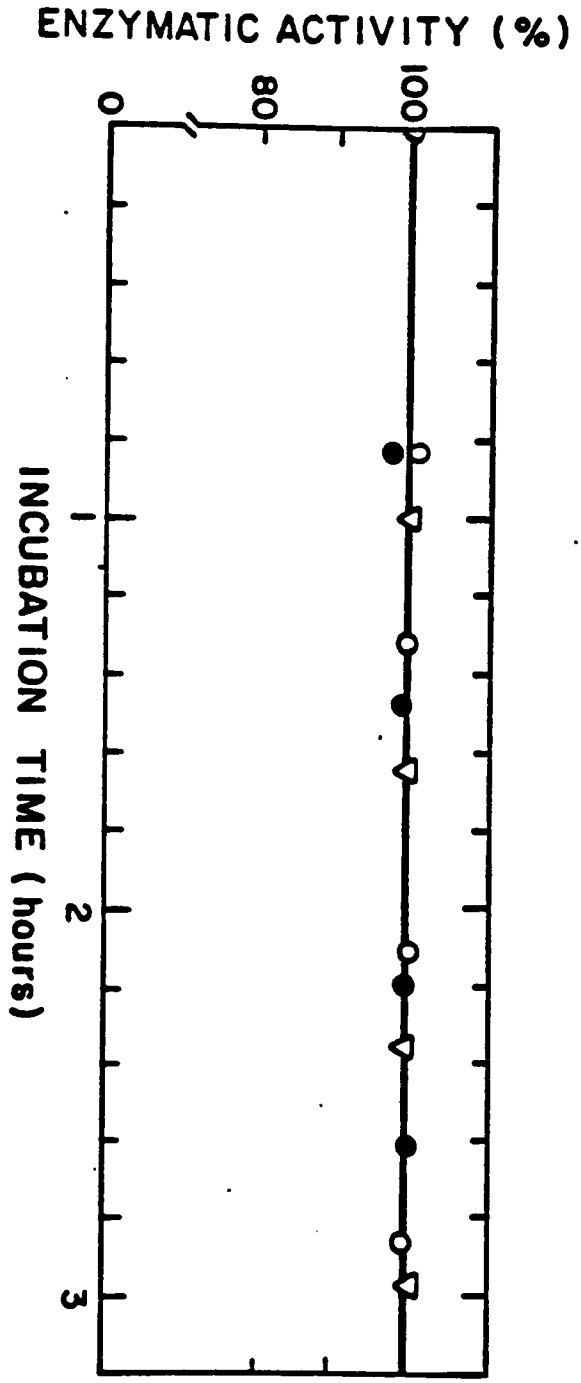


Figure 4.1

When the experiment was repeated with samples of enzyme (2 mg/ml) incubated with trypsin concentrations of 20 and 30 μg/ml for 2 hours, the same results were obtained, i.e., the overall catalytic activity remains constant. No change in the catalytic behavior of 4-aminobutyrate aminotransferase was detected when the incubation with trypsin was conducted at pH 7 and pH 7.4, (Figure 4.1). At pH 8, the aminotransferase loses a good deal of its catalytic activity when maintained at 37°C for 1 hour.

Separation of Peptides

For characterization of the action of trypsin on 4-aminobutyrate aminotransferase, the native enzyme in 0.1 M potassium phosphate buffer (pH 7) containing 0.1 mM 2-mercaptoethanol was dialyzed against 50 mM ammonium bicarbonate (pH 7.6). The dialyzed enzyme (100 mg/20ml) was incubated with 0.5 mg of trypsin at 37°C for 2 hours.

At the end of the incubation, the trypsin treated enzyme was run through Sephadex G-75 as described in experimental procedures. The fractions eluted between the excluded and included volumes of the column were combined and lyophilized. Approximately 4.6 mg of material was recovered in the lyophilized fractions. The void volume of the column (93 mg) was dialyzed against 0.1 M potassium phosphate (pH 7) containing 1 mM 2-mercaptoethanol and used for further experiments.

The lyophilized material (4.6 mg) was used for sodium dodecyl sulfate/ polyacrylamide gel electrophoresis, finger print analysis and for quantitative determination of the amino acid content.

Sodium dodecyl sulfate/polyacrylamide gel electrophoresis performed on gels containing 15% acrylamide failed to detect the presence of peptides of

molecular weight greater than 3,000 daltons. Fingerprint analysis showed the presence of at least ten spots after the cellulose sheets were sprayed with fluorescamine (Figure 4.2).

The amino acid composition of the peptides releseased by tryptic cleavage from 4-aminobutyrate aminotransferase was determined on samples hydrolyzed in 6 N HCl and in 4N methanesulfonic acid.

The results of 8 independent measurments are summarized in Table 4.1.

Basic amino acids (5 lysine, 4 arginine, 1 histidine) predominate over acidic ones (3 aspartic, 4 glutamic). The aromatic amino acids, 2 phenylalanine, 1 tyrosine and 1 tryptophan, which were detected in unhydrolyzed peptides by absorption and fluorescence spectroscopy, are also present in the hydrolyzed samples. Cysteinyl residues were not detected when unhydrolyzed samples of peptides were titrated with DTNB in the presence and absence of 6 M guanidinium-HCl. The same result (Table 4.1) was obtained by amino acid analysis of samples hydrolyzed in the presence of perfomic acid. No free amino acid could be detected on unhydrolyzed samples subjected to analysis in the amino acid analyzer.

Judging from the results included in Table 4.1, it appears that the sum of the number of amino acids yield a molecular weight of 5,500. In this context, it is interesting to note that the results of the fingerprint analysis indicate the presence of a heterogeneous population of peptides which displayed molecular weights lower than 3,000 on sodium dodecyl sulfate/ polyacrylamide gel electrophoresis.

This finding does not imply that several bonds are hydrolyzed independently from each other by the reaction with trypsin; it is possible that the cleavage

Figure 4.2

Fingerprint pattern given by the peptides released from the native enzyme. Electrophoresis was conducted at pH 4.4 in the solvent system pyridine/ acetic acid/ H_2O/ acetone (2:4:79:15 v/v/v/v). Chromatography in the second dimension was performed using the solvent system n-butanol/pyridine/acetic acid/H_2O (15:10:3:12).

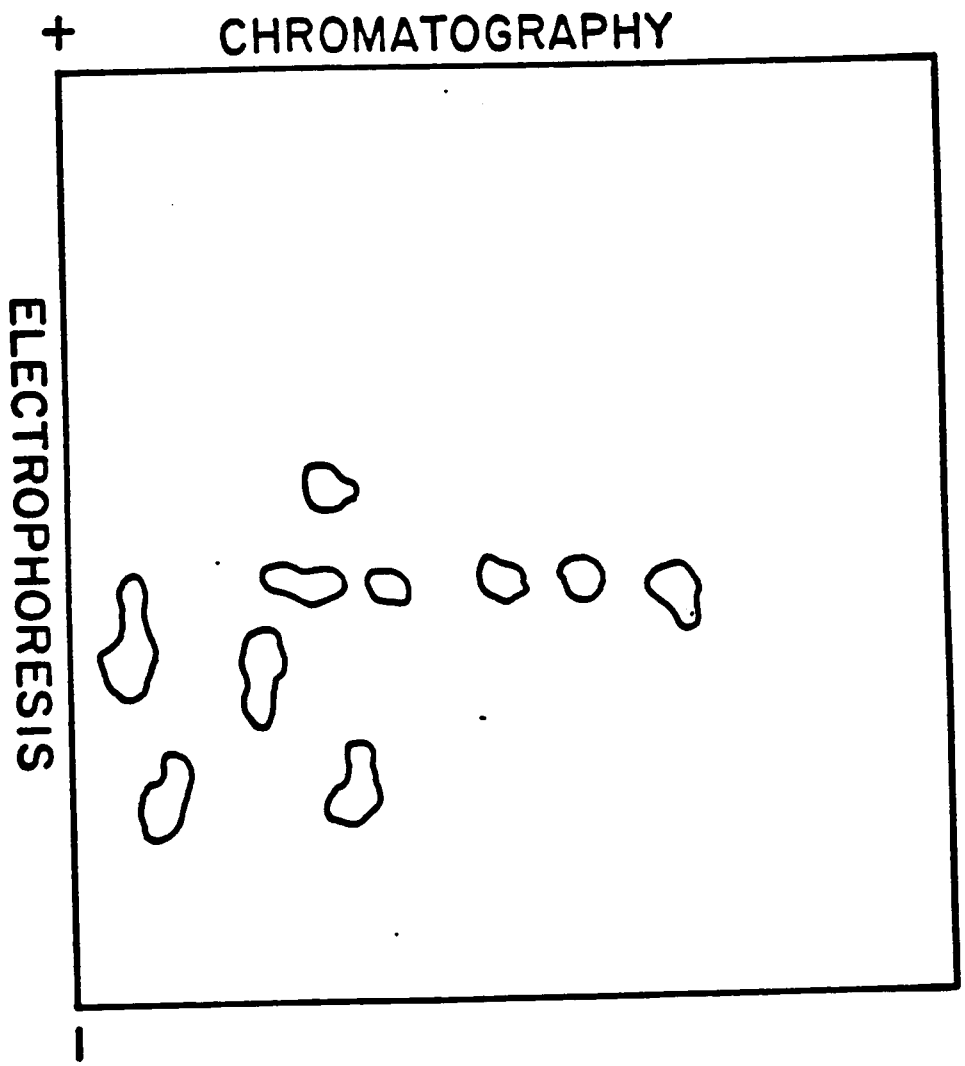


Figure 4.2

TABLE 4.1

AMINO ACID COMPOSITION OF THE PEPTIDES DERIVED FROM 4-AMINOBUTRYATE AMINOTRANSFERASE

Amino Acid	Calculated Value	Nearest Integer
Arg	4.17	4
Lys	5.30	5
His	0.84	1
Phe	1.75	2
Tyr	1.12	1
Leu	2.9	3
Ile	1.73	2
Met	1	1
Val	2.85	3
Cys[a]	0	0
Ala	2.92	3
Gly	2.80	3
Pro	3.10	3
Glu	4.05	4
Ser	1.20	1
Thr	1.10	1
Asp	2.92	3
Trp[b]	1.02	1

a: Determined from the amount of cysteic acid found in the performic acid oxidized sample, and from titrations of the unhydrolyzed material by DTNB.

b: Determined on samples hydrolyzed in 4N methane sulfonic acid.

of only one bond in the native enzyme precedes the subseqent breaking of several bonds on the released peptide of 5,500 molecular weight.

The mechanism by which trypsin cleaves the aminotransferase is the subject of further investigations in our laboratory.

Characterization of the Shortened Enzyme Derivatives

The molecular weight of the shortened enzyme derivative was determined by polyacrylamide gradient gel electrophoresis using standards of known molecular weight, i.e., bovine serum albumin, ceruloplasmin and catalase. Figure 4.3 shows the plot of the reciprocal of the migration distance (mm) of the proteins from the origin (1/XL) versus the cubic root $(MW)^{1/3}$ of the molecular weight.

The plot is linear; and from this plot the molecular weight of the shortened enzyme derivative (95,000) was found to be slightly smaller than the molecular weight of the native enzyme (100,000).

The concentration dependence of the sedimentation coefficients of native and modified enzymes were examined in the analytical ultracentrifuge over the concentration ranges 8 μM - 3 μM. (Figure 4.4).

At a concentration of 8 μM, the sedimentation coefficient of the native enzyme (5.15 S) is decreased to a value of 5.05 as a result of tryptic digestion. This reduction in sedimentation coefficeint was also detected at protein concentrations around 3 μM, the lowest concentration studied in the analytical ultracentrifuge equipped with optical scanner. As illustrated in Figure 4.4, there is little variation in the sedimentation values of both species when the concentration is decreased, indicating that none of the enzyme species tend to dissociate into subunits of smaller molecular weight at pH 7.

Figure 4.3

Determination of the molecular weight of trypsin treated 4-aminobutyrate aminotransferase on polyacrylamide gradient gel electrophoresis. XL is the migration distance (mm) of the protein from the origin. The following standards were used: catalase (1), ceruloplamin (2), bovin serum albumin (3), 4-aminobutyrate aminotransferase (5). The molecular weight of trypsin treated 4-aminobutyrate aminotransferase is 95,000 (4). Sodium dodecyl sulfate-polyacrylamide gel eletrophoresis pattern of trypsin treated 4-aminobutyrate aminotransferase. The standards of known molecular weight are myosin (200,000), phosphorylase B (92,500), ovoalbumin (43,000), α-chymotrypsinogen (25,700) and cytochrome C (12,300).

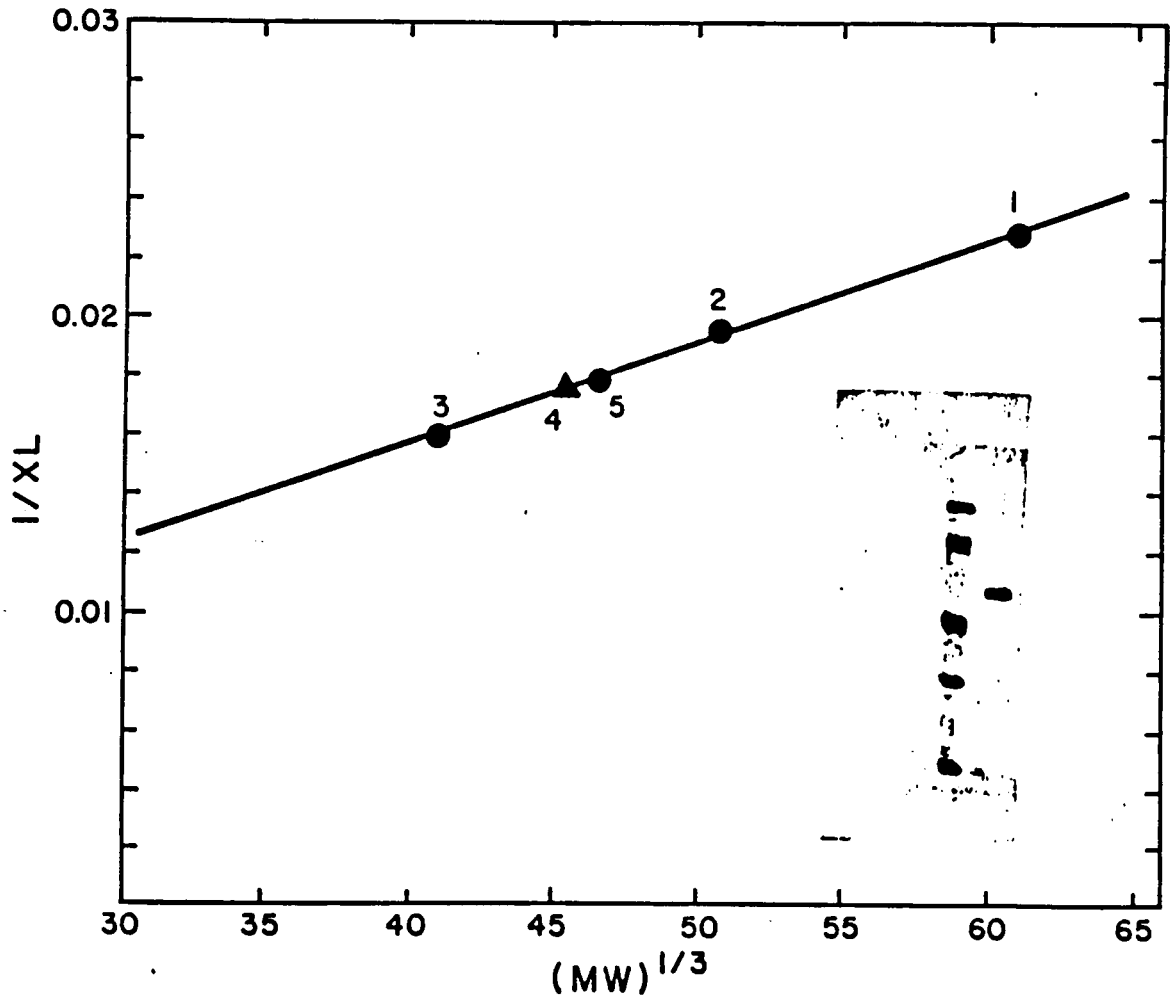


Figure 4.3

Figure 4.4

Sedimentation coefficients of native (●) and trypsin treated (0) enzymes at pH 7, 0.1M potassium phosphate. The ultracentrifuge was operated at 60,000 RPM.

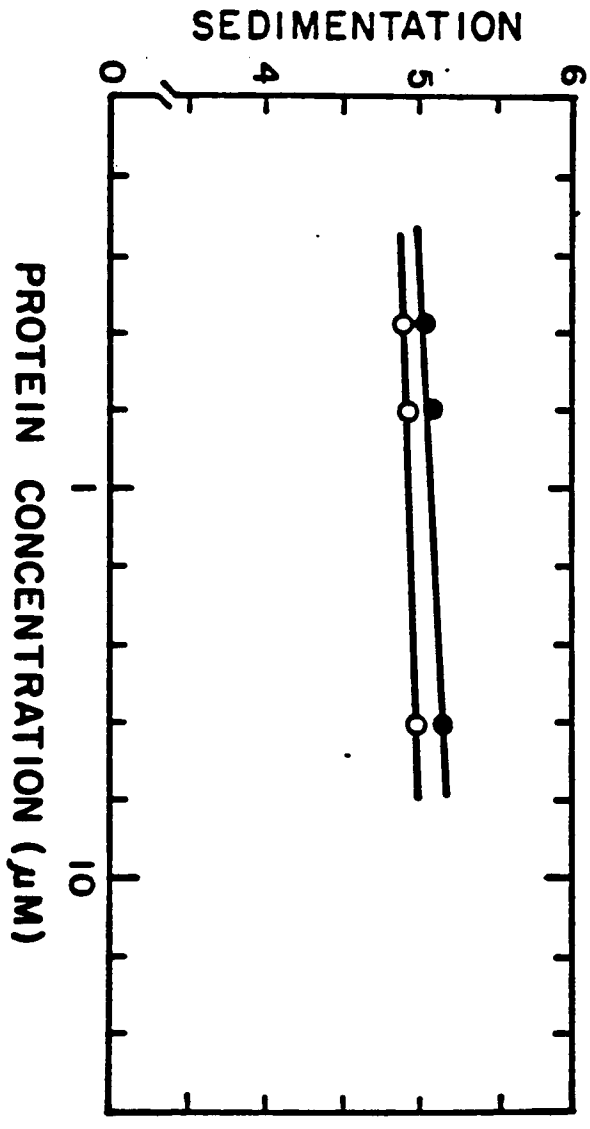


Figure 4.4

Catalytic Properties

The absorption spectra of the shortened enzyme derivative displayed the characteristic features of the cofactor pyridoxal-5-P covalently bound to the catalytic site. Two absorption bands centered at 330 and 415 nm were detected over the spectral range 300-500 nm at pH 5.8, pH 6 and pH 7.0. The intensity of the absorption bands are not affected by variations in the pH of the solution.

The specific activity of the modified enzyme is, within experimental error, identical to that of the native enzyme. Addition of pyridoxal-5-P, followed by preincubation at 25°C for 1 hour prior to enzymatic assays, did not enhance the specific activity of the shortened enzyme derivative. Hence, the modified enzyme behaves as the native aminotransferase and limited proteolysis did not abolish the apparent negative cooperativity between subunits.

The results of these experiments are summarized in Table 4.2.

Reactivity of Sulfhydryl Groups

4-Aminobutyrate aminotransferase is easily inactivated by DTNB; the extent of chemical modification was measured by monitoring the release of 2-nitro-5-mercaptobenzoate at 412 nm. Under denaturing conditions, five SH groups/dimer are titrable with DTNB (23). The time course for a typical reaction under pseudo-frist order conditions is given in Figure 4.5.

The reaction of approximately 1.2 sulfhydryl groups/dimer proceeds with an observed rate constant of 0.05 min^{-1} leading to 90% loss of catalytic activity (Figure 4.5). Addition of 2-mercaptoethanol (1 mM), followed by dialysis against 0.1 M potassium phosphate (pH 7) containing 1 mM 2-mercaptoethanol, readily restores 90% of the original enzymatic activity (results not shown).

TABLE 4.2

CATALYTIC PROPERTIES OF NATIVE AND TRYPSIN-TREATED 4-AMINOBUTYRATE AMINOTRANSFERASE.

Enzyme Samples	Absorption [a] (λ nm)	pH	Specific Enzyme Acitivity[b] (units/mg)
Native	330,415	7	20
Trypsin-Treated	330,415	7	20
Native	330,415	5.8	20
Trypsin-Treated	330,415	5.8	19.2
Native + PLP[c]	—	—	20
Trypsin-Treated + PLP[c]	—	—	20.2

a: Absorption spectra recorded at protein concentrations of 5 mg/ml at the indicated pH values.

b: Specific activity of the enzymes exposed to the indicated pH values and assayed at pH 8.4.

c: Samples preincubated with 20 fold molar excess of pyridoxal-5-P prior to enzymatic assays.

Figure 4.5

Time course of reaction of DTNB with thiol groups of 4-aminobutyrate aminotransferase (5 μM) (0) and the trypsin treated enzyme (5 μM) at 25°C in 0.1 M potassium phosphate (pH 7) (●). The concentration of DTNB was 150μ M. Loss of catalytic activity of the native enzyme as a function of incubation time with DTNB. (□)

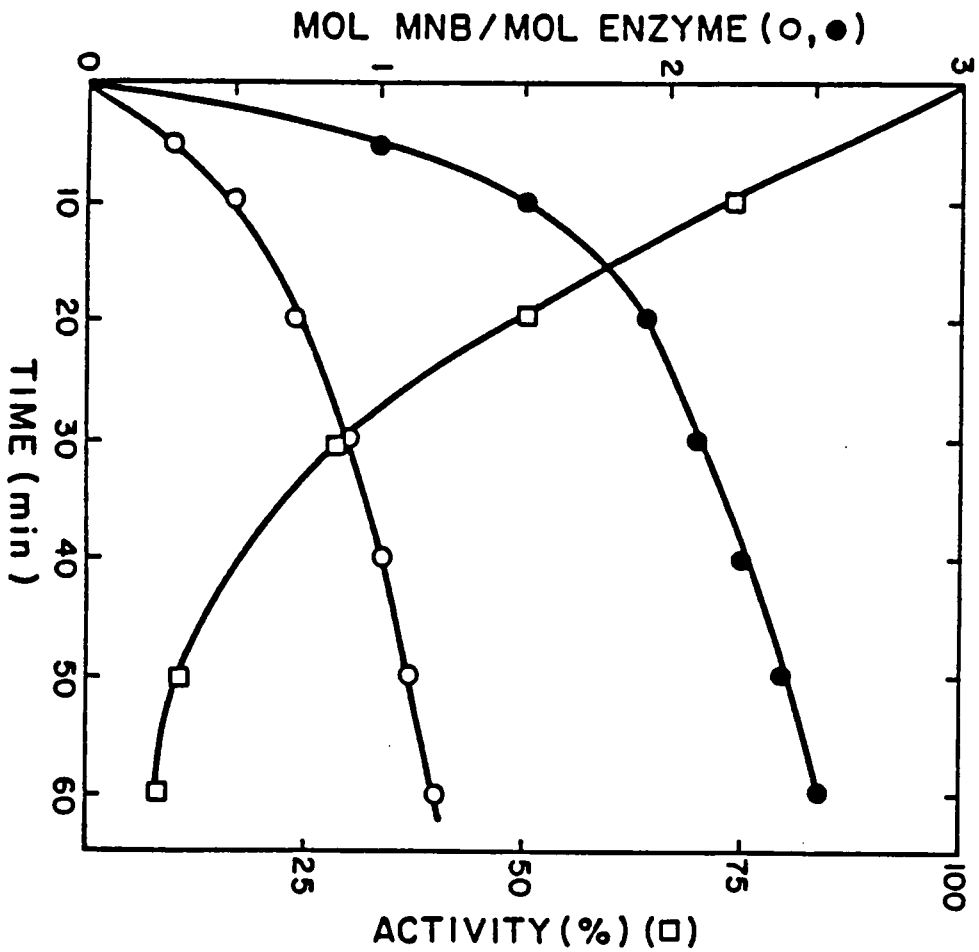

MOL MNB / MOL ENZYME (o, ●)
0
1
2
3
10
20
30
40
50
60
TIME (min)
25
50
75
100
ACTIVITY (%) (□)

Figure 4.5

This well behaved kinetic system can be exploited to probe the reactivity of the sulfhydryl groups of the shortened enzyme derivative.

When the trypsin treated enzyme was allowed to react with DTNB under pseudo first order conditions, the reaction of approximately 2.5 sulfhydryl groups/mol of enzyme has taken place with an observed rate constant of 0.08 min^{-1}.

This increased accessibility of SH groups to the attacking reagent DTNB reflects local conformational changes of the enzyme elicited by tryptic cleavage.

Fluorescence Polarization

Protein conformational changes elicited by trypsin action were examined using a fluorescent probe covalently linked to the protein.

6-Iodoacetamido fluorescein (IAF) was selected for these studies for two reasons: First, because it displays absorption and emission properties distinct from the cofactor pyridoxal-5-P; and second, because IAF is a potential modifier of sulfhydryl groups which are not released during proteolytic cleavage.

In marked contrast to DTNB, IAF and iodoacetamide have no effect on the catalytic activity of the aminotransferase. The enzyme was reacted with IAF under the conditions described in experimental procedures. The labelled enzyme exhibits an absorption band at 495 nm and emission band centered at 525 nm which can be unambiguosly assigned to fluorescein (Figure 4.6). Using an exctinction coefficient of $3.4 \times 10^{4} M^{-1} cm^{-1}$ for bound fluorescein, a degree of labelling of 0.9 chromophores/dimer was determined for the reacted enzyme.

Figure 4.6

Changes in the polarization of fluorescence (●) and fluorescence intensity (0) of IAF- aminotransferase (2 mg/ml) incubated with trypsin (40 μg/ml) at 25°C. Insert, emission spectra of IAF-aminotransferase before (2) and after (1) trypsin digestion.

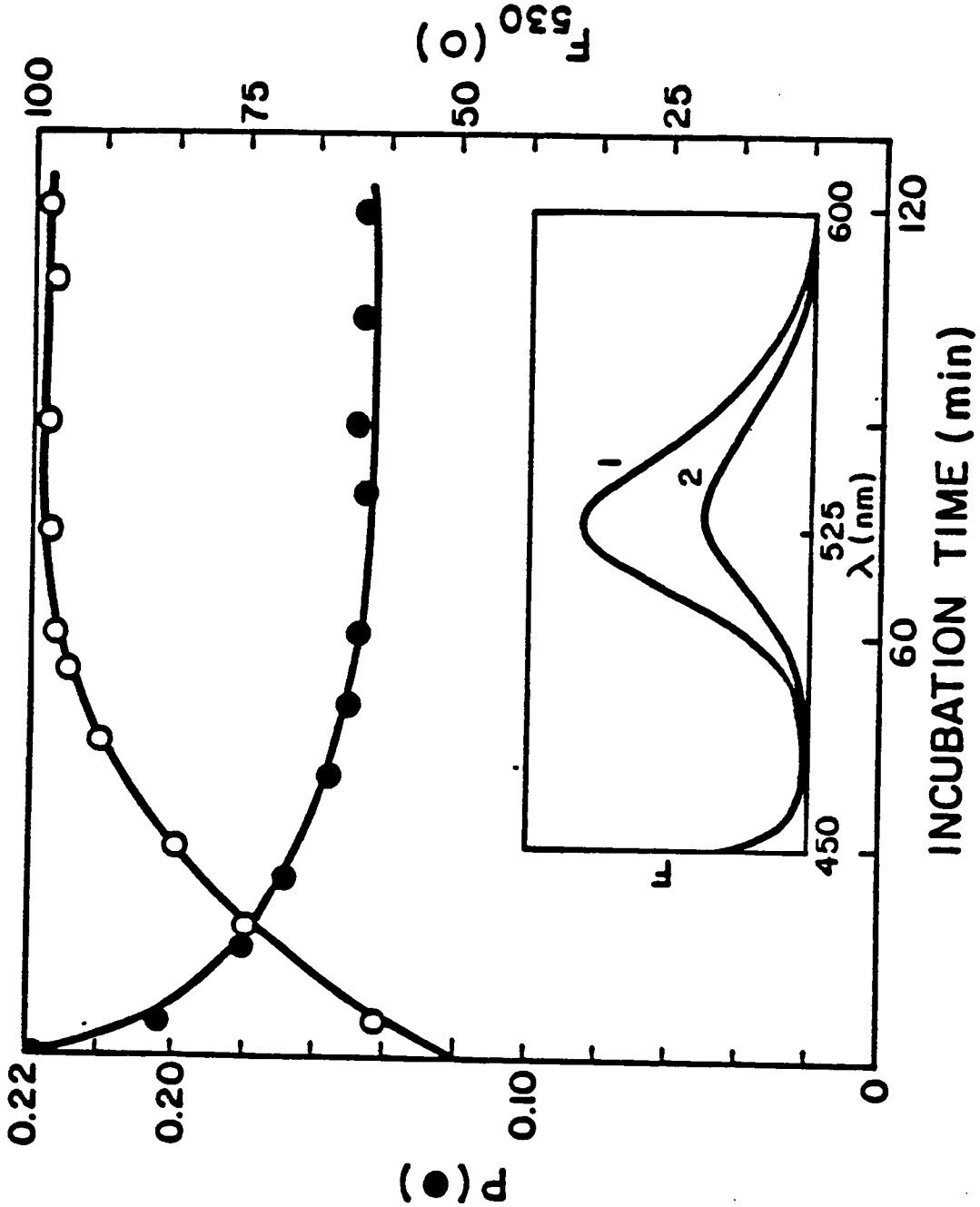

100
75
50
25
0
F_{530} (○)
0.22
0.20
0.10
P (●)
60
120
INCUBATION TIME (min)
F
450
525
600
λ (nm)
1
2

Figure 4.6

In order to ascertain whether IAF has blocked sulfhydryl groups, samples of native and IAF aminotransferase were allowed to react with DTNB under denaturing conditions (6M guanidinium-HCl).

The results of those measurements indicated that 5 SH groups/dimer of native enzyme are blocked by DTNB, whereas only 3.9 SH/dimer of IAF-aminotransferase have reacted with DTNB (Table 4.3). Thus, these results support the concept that there is one class of sulfhydryl groups in the enzyme which can be modified by the alkylating reagent IAF without any concommitant loss of catalytic acitivity. The spectroscopic properties of bound fluorescein can be used conveniently to detect large structural fluctuations induced by reaction with trypsin. When IAF-aminotransferase (2 mg/ml) was incubated with trypsin (40 μg/ml) at pH 7, and the polarization values recorded as a function of time, the results included in Figure 4.6 were obtained. The initial polarization of IAF-aminotransferase (p = 0.22) is decreased to a value of p =0.15 after 60 minutes incubation at 25°C. As shown in Figure 4.6, an increase in the fluorescence intensity was also observed as a function of time.

Both parameters, i.e. fluorescence intensity and polarization of fluorescence, reach constant values after 1 hour incubation at 25°C.

The changes in polarization and fluorescence intensity might be due to the release of either free dye or small peptides tagged with fluorescein. To examine this possibility, the mixture (2.5 ml) containing IAF-aminotransferase and trypsin was passed through a small column of Sephadex G-25 (1 x 25 cm) equilibrated with 0.1 M potassium phosphate (pH 7) containing 1 mM 2-mercaptoethanol. The protein eluted in the void volume of the column was labelled with fluorescein and displayed a specific activity of 19.2 units/mg of protein (Table 4.3).

TABLE 4.3

CATALYTIC AND STRUCTURAL PROPERTIES OF TRYPSIN DIGESTED IAF-AMINOTRANSFERASE

(0.9 mol IAF/mol of enzyme)

Enzyme Sample	Number of SH/ mol of Enzyme[a]	Lifetime (ns)	Polarization	Rotational Relaxation Time (ns)	Catalytic Activity (units/mg)
IAF-Enzyme	3.9	2.7	0.22	106	20
Trypsin Digested IAF-Enzyme[b]	3.8	4	0.15	33	19.2[c]

a: Titrated with DTNB in 6M Guanidinum-HCl at pH 7.

b: IAF-aminotransferase (2 mg/ml) incubated with trypsin (40μg/ml) at 25°C for 1 hour at 25°C in 0.1M potassium Phosphate (pH 7). At the end of the incubation, the digested sample was run through Sephadex G-25 column equilibrated with 0.1M potassium phoshpate (pH 7). The protein eluted in the void volume of the column was used for SH titration and polarization measurements.

c: Enzymatic activity determined on the shortened enzyme derivative after passage through a Sephadex column G-25 (1 x 15 cm) equilibrated with 0.1 M potassium phosphate (pH 7) containing 1 mM 2-mercaptoethanol.

No free dye or small peptide tagged with fluorescein could be detected on the fractions collected after elution of the protein by fluorescence measurements at 530 nm (excitation 490 nm). These results demonstrate that changes in the luminescence parameters cannot be related to the release of small molecular weight peptides tagged with fluoresceine.

When the polarization values of IAF-aminotransferase and trypsin digested IAF-aminotransferase were recorded as a function of temperature, the plots of $1/p - 1/3$ versus T/η were found to be linear in the temperature range 6-25°C (Figure 4.7).

Taking τ, the fluorescence lifetimes of bound fluoresceine to be 2.7 and 4 ns (Table 4.3) for IAF-aminotransferase and trypsin digested IAF-aminotransferase, respectively, the rotational relaxation time of IAF-aminotransferase is three fold greater than the corresponding value of the digested protein (Table 4.3). A rotational relaxation time ratio ($\rho N/\rho T$) of 3, as obtained from steady-state polarization measurements, indicates flexibility of the polypeptide chains with enhanced rotational freedom of the probe covalently attached to sulfhydryl groups of the protein.

Figure 4.7

Plots of 1/P - 1/3 versus T/η for IAF-aminotransferase (●) and trypsin treated IAF - aminotransferase (0) in 0.1 M potassium phosphate (pH 7). Rotational relaxation times of 106 nanoseconds and 33 nanoseconds were obtained for native and trypsin treated enzymes, respectively.

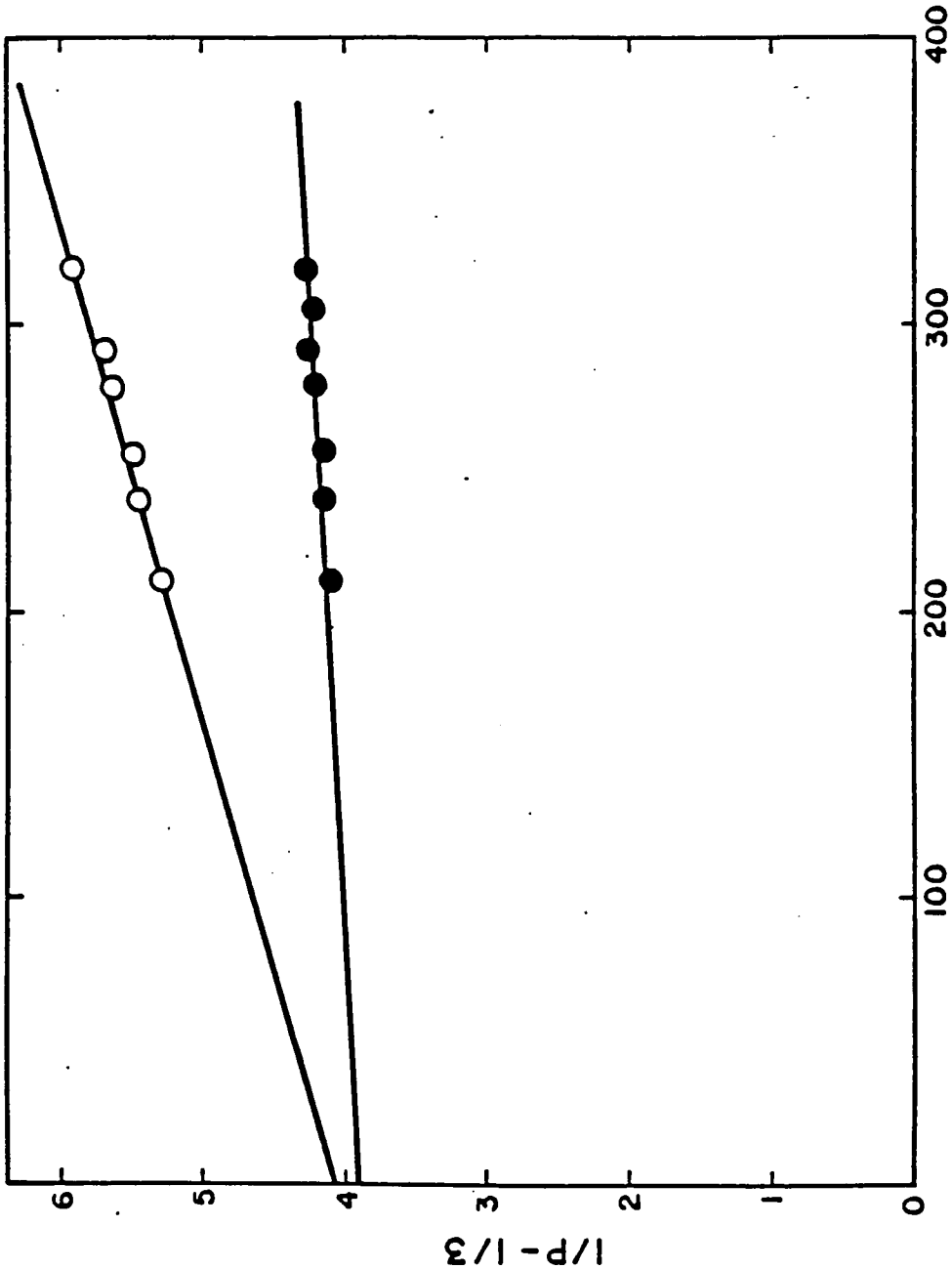


Figure 4.7

III. DISCUSSION

The results reported in this paper have direct bearing on two questions a) the origin of different forms of mitochondrial 4-aminobutyrate aminotransferase and b) the effect of conformational fluctuations on the catalytic parameters of the enzyme.

Recently, the subject of limited proteolysis of aminotransferase have attracted the attention of two independent laboratories. Hargrove et al.(60) have shown that the endopeptidase cathepsin-T generates multiple forms of tyrosine aminotransferase. Upon incubation of tyrosine aminotransferase-I with cathepsin, tyrosine aminotransferase II and a peptide of about 4,500 daltons are generated without release of a single amino acid.

On the other hand, Sandmeier and Christen (61) have reported that mitochondrial aspartate aminotransferase is cleaved selectively by trypsin at two peptide bonds yielding enzyme derivatives devoid of catalytic activity.

The studies reported in this paper have demonstrated that limited proteolysis of 4-aminobutyrate aminotransferase does not impair its catalytic function.

As a consequence of tryptic digestion, a heterogeneous population of small molecular weight peptides is released from the native enzyme. The mechanism by which trypsin cleaves the aminotransferase remains unclear on the basis of the present data, and it is the subject of further investigations aiming at the elucidation of the sequence of the COOH and NH_2 terminus of the enzyme.

The shortened enzyme derivative generated by trypsin action is not only catalytically competent, but also preserves negative cooperativity between subunits. In this connection, it should be noted that the presence of multiple forms of the enzyme has been reported by several laboratories (6,14). In the light of our studies performed on the shortened enzyme derivative generated by trypsin digestion of 4-aminobutyrate aminotransferase from pig brain, it seems reasonable to suggest that the multiple forms of this enzyme are derived from a common protein precursor. Research conducted in several laboratories indicate that protein molecules display conformational fluctuations in the nanosecond, millisecond and second time ranges (62,63). More frequently considered are those fluctuations where a given structural part of the macromolecule is destroyed and a new configuration is built up. The shortened enzyme derivative of 4-aminobutyrate aminotransferase can be used to investigate the effect of large conformational fluctuations on the catalytic power of an enzyme.

The results obtained using chemical and biophysical methods indicate that conformational changes have taken place in 4-aminobutyrate aminotransferase as result of trypsin action. Thus, the increased reactivity of thiol groups toward DTNB reflects structural fluctuations in the microenvironment surrounding sulfhydryl residues critically connected with catalytic activity.

On the other hand, the spectroscopic properties of a fluorescent probe covalently linked to another class of sulfhydryl groups are influenced by local conformational changes in the protein.

The polypeptide chains of the shortened enzyme derivative display high degree of flexibility as revealed by polarization of fluorescence measurements.

Indeed, the decrease in rotational relaxation time from 106 to 33 ns, the values corresponding to native and trypsin digested enzymes, respectively, is more that can be expected for macromolecules exhibiting such small differences in molecular weight.

These conformational changes, however, did not prevent the development of the steps required for activation of the enzyme substrate complex, suggesting that structural fluctuations detected at the level of sulfhydryl groups did not perturb the catalytic site domain.

CHAPTER V

PRIMARY STRUCTURE OF THE CATALYTIC SITE OF 4-AMINOBUTYRATE AMINOTRANSFERASE

Amino acid sequencing studies near the cofactor binding site for PLP-enzymes have been carried out in many laboratories (64,65,66,67). A significant sequence homology near the cofactor binding site of phosphorylases isolated from different sources was demonstrated by Fischer et al (68,69,70). In case of pig heart aspartate aminotransferase, PLP is found at exactly same position, which is Lys 258, either in cytosolic or in mitochondrial enzyme as well as with great similarity of amino acid sequence between the PLP-containing peptides (71,72).

It seems to be worthwhile to determine the primary structure of PLP-binding peptide of 4-aminobutyrate aminotransferase since we have reported information about the properties of catalytic site of the enzyme (25,50,51,73).

In this chapter, the sequence of 20 amino acids including the lysine residue that binds PLP in 4-aminobutyrate aminotransferase is discussed. In addition, N-terminal sequence (16 amino acids) and C-terminal analysis of the enzyme are also presented.

I. EXPERIMENTAL

TPCK-trypsin was purchased from Worthington, chymotrypsin from Boehringer, thermolysin from Serva, and Staphylococcus aureus protease from Miles Lab. Sephadex G-25, G-50 and SP-Sephadex were obtained from

Pharmacia. Polygram CEL 400 cellulose sheets were products of Macherey and Nagel. Fluorescamine was from Hofmann La Roche. P-Pyridoxyl lysine was synthesized following the procedure of Schnackerz and Noltmann (74). All other chemicals were of analytical grade from Merck.

Isolation of Phosphopyridoxyl Peptide From 4-Aminobutyrate Aminotransferase

Purification of 4-aminobutyrate aminotransferase from pig brain was performed by a method as described previously (73).

75 mg of 4-aminobutyrate aminotransferase in 0.1 M triethanolamine buffer, pH 7.0 was reduced with $NaBH_4$, dialyzed against 50 mM NH_4HCO_3, and then the pH was adjusted to 8.0 with formic acid. The reduced enzyme was incubated with 500 µg TPCK-trypsin at 35°C for one hour followed by denaturation in boiling water bath. After cooling it down to room temperature, additional 500 µg TPCK-trypsin was used to digest the denatured protein under the same condition. The tryptic digest was concentrated to 5.0 ml using rotary evaporator, and centrifuged. The soluble supernatant was applied to a column of Sephadex G-50 SF (1 x 300 cm) equilibrated with 100 mM ammonia. Phosphopyridoxyl peptide-containing fractions were analyzed by finger print technique on cellulose thin layer sheets (CEL 400) and by amino acid analysis. The phosphopyridoxyl peptide was visualized under ultraviolet light, and the other peptides were detected by spraying with 0.01% (w/v) fluorescamine in acetone. The cellulose sheets were sprayed with 10% (v/v) triethylamine in dichloromethane before and after the fluorescamine spraying.

The fractions containing phosphopyridoxyl peptide were combined, dried by nitrogen gas, and then redissolved in 0.01 M pyridine-HCOOH, pH 3.0. The peptides were applied to a column of SP-Sephadex (1 x 20 cm) and eluted by

a gradient buffer made of 50 ml of each of the following pyridine-HCOOH: 0.03M, 0.04M, 0.06 M, 0.08 M, 0.16 M and 0.32 M pyridine adjusted to pH 3.0, 3.1, 3.3, 3.5, 4.1, and 4.6 with HCOOH, respectively. At the end of elution, the second gradient composed of 0.32 M (50 ml) and 1.0 M (50 ml) pyridine-HCOOH buffer, pH 4.6 was used to elute remaining peptides. Small aliquot of each fraction was dried and analyzed by thin layer chromatography on cellulose sheets (CEL 400) in a solvent system pyridine/n-butanol/acetic acid/water (10/15/3/12, v/v/v/v). Homogeneous fractions of phosphopyridoxyl peptide were pooled for sequence determination.

Fingerprint Technique and Amino Acid Analysis

Peptide finger-prints on cellulose sheets (CEL 400) were made according to the procedure of Schiltz and Reinbolt (57). Peptides on cellulose sheets were isolated by scraping off the spots into Pasteur pipettes plugged with glass fiber filter. The peptides were eluted with 600 μ l of 6N HCl followed by hydrolysis for 20 hours at 100°C in sealed tubes. Amino acid analyses for the hydrolyzed samples were performed on Biotronik amino acid analyzer, model LC6000E.

Sequence Determination

N-terminal sequence of 4-aminobutyrate aminotransferase and sequence of the isolated phosphopyridoxyl peptide were determined on Sequemat model 12-S solid-phase sequenator. Either the peptide or the protein sample in 0.1 M pyridine-HCl, pH 5.8 was attached to inert glass support by reacting the sample with 300 mg of N-(2-aminoethyl)-3-aminopropyl glass (AEAPG) and 5 mg of N-ethyl-N-dimethyl aminopropylcarbodiimide (EDC) at 40°C for 1 hour

while stirring. Immediately after the reaction, the suspension was washed three times each with water, methanol and diethylether followed by air-drying. Phenylthiohydantoin derivatives of amino acids were identified by high performance liquid chromatography, Hewlett Packard model 1084 B.

II. RESULTS

Sequence of Phosphopyridoxyl Peptide

According to preliminary digestion of 4-aminobutyrate aminotransferase with trypsin, thermolysin, chymotrypsin and Staphylococcus aureus protease, tryptic digestion was chosen for the isolation of phosphopyridoxyl peptide.

Tryptic peptide containing phosphopyridoxyl lysine was isolated from $NaBH_4$-reduced 4-aminobutyrate aminotransferase by combination of gel filtration and ion-exchange chromatography.

Fractionation of the tryptic digest of 4-aminobutyrate aminotransferase on a column of Sephadex G-50 SF (1 x 300 cm), in which the elution was monitored at 325 nm, gave three elution peaks (Fig 5.1). However only one of them was revealed to contain phosphopyridoxyl lysine by amino acid analysis. Finger-print analysis indicated that the sample contains heterogeneous peptide species. Therefore further fractionation of the sample was performed on a SP-Sephadex column (1 x 20 cm) by eluting the peptides with pyridine-HCOOH gradient buffer. About 120 nmol of phosphopyridoxyl peptide separated by the cation exchange chromatography was judged to be pure since the sample migrated as a single fluorescent spot under ultraviolet light on CEL 400 chromatogram. Amino acid composition of the peptide was Pro (1), Asx (3), Thr (1), Ser (1), Glx (2), Gly (2), Ala (3), Val (1), Met (1), Leu (1), Phe (2), Lys (1), Lys(PXY) (1), and His (2).

Figure 5.1

Elution profile of phosphopyridoxyl peptide from Sephadex G-50SF column(1 x 300 cm). Phosphopyridoxyl-lysine was found only from peak 1.

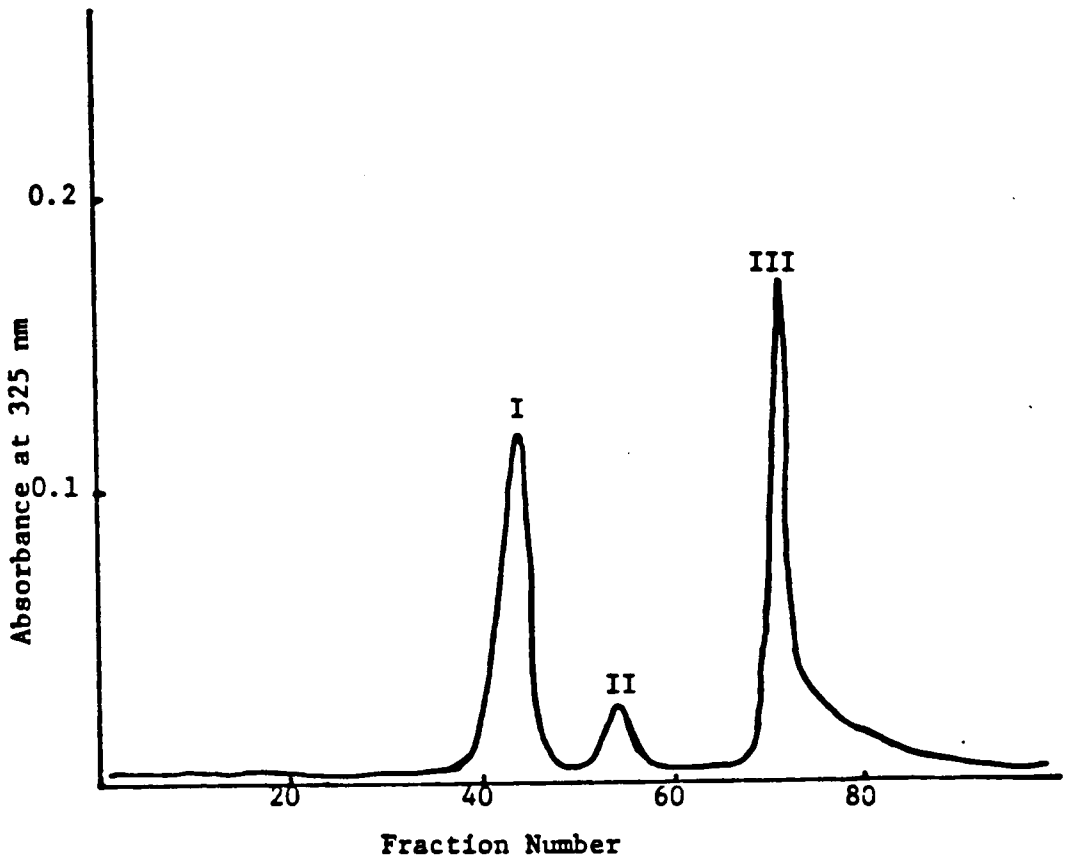


Figure 5.1

Sequence of the peptide determined by automated solid-phase technique (Fig. 5.2) was Phe-Trp-Ala-His-Glu-His-Trp-Gly-Leu-Asp-Asp-Pro-Ala-Asp-Val-Met-Thr-Phe-Ser-Lys (PXY), which fits well into the amino acid analysis.

Digestion of the phosphopyridoxyl peptide (30 n mol) with thermolysin yielded a peptide fragment composed of Lys, Lys (PXY), Phe and Ser which corresponds to C-terminal region of the above sequence. (Fig. 5.3)

N-Terminal Sequence and C-Terminal Analysis of 4-Aminobutyrate Aminotransferase

Native 4-aminobutyrate aminotransferase (8 mg) was also attached to AEAPG by EDC technique at pH 5.8 as described in Experimental. N-Terminal sequence determination up to 16 cycles was possible by automated Edman degradation, and following N-terminal amino acid sequence was obtained by duplicate experiments: Ile-X-Gln-Ala-Ala-Ala-Lys-Val-Asp-Val-Glu-Phe-Asp-Tyr-Asp-Gly (X, failed to determine).

4-Aminobutyrate aminotransferase was digested with carboxypeptidase A & B in 50 mM N-ethylmorpholine buffer, pH 8.0 at 35°C for 30 minutes followed by the addition of concentrated HCOOH to stop the reaction against a control in which the enzyme was incubated with HCOOH-treated carboxypeptides A & B. After spinning down the precipitated proteins, amino acid analyses were performed for soluble fractions. The C-terminal composition was found to be Phe, Thr, and Ala.

Figure 5.2

The solid-phase sequencing procedure.

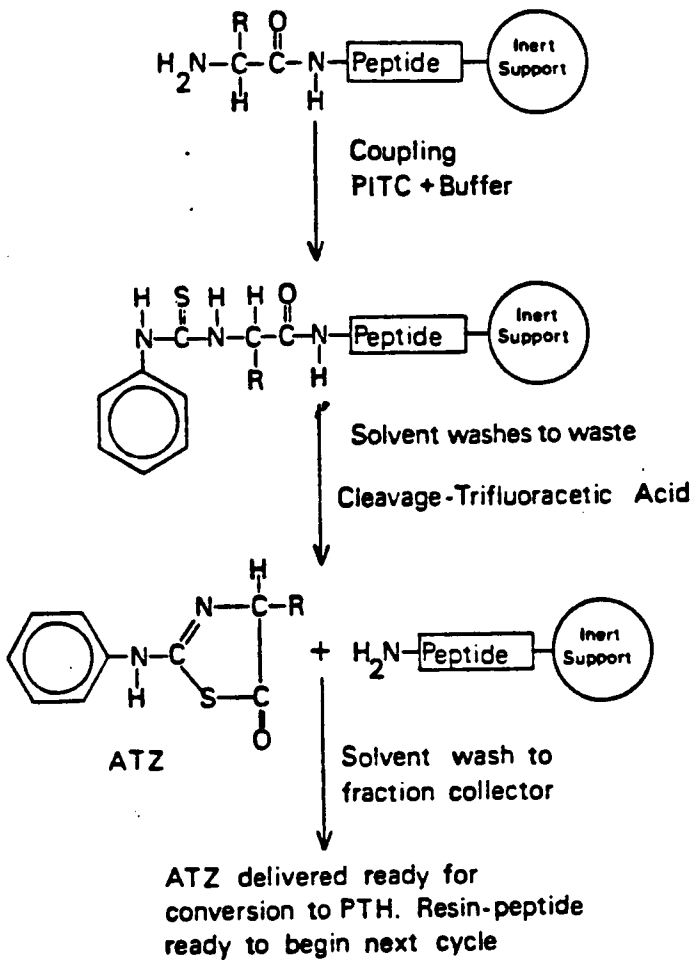

Peptide
Inert Support
Coupling
PITC + Buffer
Peptide
Inert Support
Solvent washes to waste
Cleavage-Trifluoracetic Acid
ATZ
+
Peptide
Inert Support
Solvent wash to
fraction collector
ATZ delivered ready for
conversion to PTH. Resin-peptide
ready to begin next cycle

Figure 5.2

Figure 5.3

Finger-print for thermolytic fragments of phosphopyridoxyl peptide isolated from 4-aminobutyrate aminotransferase. Electrophoresis in pyridine/acetic acid/H_2O/acetone(2/5/79/15,v/v/v/v, pH 4.4) and chromatography in n-BuOH/pyridine/acetic acid/H_2O(15/10/3/12,v/v/v/v). Spot 1 contains Lys(1), Lys(PXY)(1), Phe(1) and Ser(1).

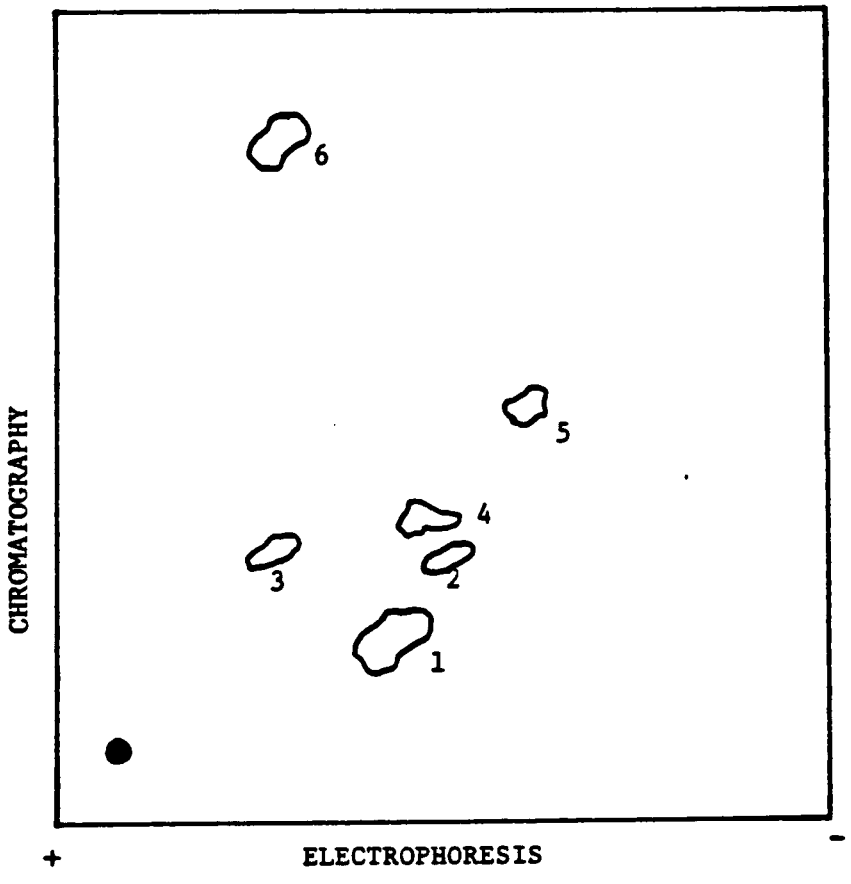


Figure 5.3

III. DISCUSSION

Phosphopyridoxyl peptide of 20 amino acids isolated from pig brain 4-aminobutyrate aminotransferase by tryptic digestion was revealed to be relatively hydrophobic.

Sequence comparison of the peptide with pig heart cytosolic or with mitochondrial aspartate aminotransferase (72) indicates that there is no significant structural homology (Table) although a partial sequence of-Phe-Ser-Lys (PXY) of the peptide is identical with that of cytosolic aspartate aminotransferase, which is unusual in that 4-aminobutyrate aminotransferase is a mitochondrial enzyme. (Table 5.1)

N-terminal amino acid (Ile) of 4-aminobutyrate aminotransferase from pig brain turned out to be consistent with that of the enzyme from rat brain as reported by Maitre et.al., (10). The enzyme could be easily attached to AEAPG in the absence of denaturing agent, and Edman degradation occured in good yield up to 16 cycles to give a strong sequence indicating that the N-terminus of the enzyme is exposed to the surface of the protein molecule, and each monomer has essentially identical N-terminus chain of 16 amino acids suggesting that two subunits are homologous polypeptide chains.

TABLE 5.1

AMINO ACID SEQUENCES OF PHOSPHOPYRIDOXYL PEPTIDES

Enzyme	Sequence
GABA-T[a]	-Phe-Trp-Ala-His-Glu-His-Trp-Gly-Leu-Asp-Asp-Pro-Ala-Asp-Val-Met-Thr-Phe-Ser-Lys(PXY) (Ala)
s-ATT[b]	-Ala-Ile-Arg-Tyr-Phe-Val-Ser-Glu-Gly-Phe-Glu-Leu-Phe-Cys-Ala-Gln-Ser-Phe-Ser-Lys(PXY)
m-ATT[c]	-Ala-Val-Arg-His-Phe-Ile-Glx-Glx-Gly-Ile-Asn-Val-Cys-Leu-Cys-Gln-Ser-Tyr-Ala-Lys(PXY)
D-SD[d]	-Ile-Ser-Gly-Gln-Leu-Leu-Leu-Lys-Lys-Asp-Ser-His-Leu-Pro-Ile-Ser-Gly-Ser-Ile-Lys(PXY)

a: 4-aminobutyrate aminotransferase from pig brain
b: cytosolic aspartate aminotransferase from pig heart
Barra et al. FEBS Letters (1977), 83, 241
c: mitochondrial aspartate aminotransferase from pig heart
Barra et al. FEBS Letters (1977), 83, 241
d: D-serine dehydratase from E. coli
Schiltz et al FEBS Letters (1981), 134, 57

CHAPTER VI

GENERAL CONCLUSION

Homogeneous 4-aminobutyrate aminotransferase (M.W. 100,000) is a dimeric protein consisting of two subunits of identical molecular size. The purified holoenzyme exhibits k_{cat} of 9.6 sec^{-1} and contains 1 molecule of pyridoxal-5-phosphate per dimer with a dissociation constant of 1 nM. An additional cofactor molecule binds to the holoenzyme with a dissociation constant of 3 μM without changin the k_{cat} and K_m of the transaminase.

The catalytic function of 4-aminobutyrate aminotransferase is lost after chemical modification of 0.5 mol enzyme-bound cofactor per mol dimer with the suicide substrate gabaculine in which the reaction yields m-carboxyphenyl pyridoxamine-5-phosphate. Reversible binding of the adduct to the transaminase was demonstrated by coupling with pyridoxine-5-phosphate oxidase which converts m-carboxyphenyl pyridoxamine-5-phosphate to pryidoxal-5-phosphate. Derivatization of the enzyme saturated with pyridoxal-5-phosphate (2 mol/mol dimer) with gabaculine results in the incorporation of about 1 molecule m-carboxyphenyl pyridoxamine-5-phosphate per dimer with concomitant loss of catalytic activity. These results suggest the presence of two potential binding sites on dimeric structure. It was also shown that the binding of additional cofactor molecule to the holoenzyme does not affect the kinetic parameters such as k_{cat} and K_m. The maximum catalytic activity is expressed with one molecule pyridoxal-5-phosphate per dimer, and the second binding site of the enzyme becomes functional only after the modification of the cofactor tightly bound to the enzyme. In this respect, 4-aminobutyrate aminotransferase exhibits different catalytic behavior than aspartate aminotransferase, which gives

maximum enzymatic activity after the saturation of two binding sites with pyridoxal-5-phosphate.

4-Aminobutyrate aminotransferase is inactivated by modification of 6 lysyl residues per dimer with o-phthalaldehyde. The inactivation is not due to the release of enzyme-bound cofactor. Preincubation of the enzyme with 2-oxoglutarate completely protects the enzyme against the inactivation while excess pyridoxal-5-phosphate failed to exert the protection effect. These results indicate that lysyl residues other than those involved in the binding of cofactor directly interact with carboxyl groups of the substrate 2-oxoglutarate.

The cofactor analogue bis-pyridoxal-5-phosphate reacts with two lysyl residues characterized by low pK values (pK = 7.3) on 4-aminobutyrate aminotransferase at a slow rate (k_{obs} = 0.03 min^{-1}). The modification leads to irreversible loss of catalytic activity, but the protection afforded by the substrate 2-oxoglutarate against the inactivation which implies that binding of 2-oxoglutarate is the major factor contributing to stabilize the catalytic binding site. Several lines of experimental evidence indicate that the inactivation is not related to dissociation of the cofactor from the catalytic site. A possibility that the bifunctional reagent is involved in the cross-linking of lysyl residues located on different subunits was excluded by sodium dodecyl sulfate polyacrylamide gel electrophoresis.

4-Aminobutyrate aminotransferase undergoes limited proteolysis by incubation with trypsin, yielding catalytically intact species and fragments of 5,500 molecular weight consisting of about 10 small peptides. Significant changes in protein conformation induced by trypsin digestion were detected on iodoacetamidofluorecein-labeled enzyme. In addition, more thiol groups on the transaminase become reactive toward Ellman's reagent DTNB as a result of

tryptic cleavage, but the catalytic function remained intact. These results strongly indicate that multiple forms of 4-aminobutyrate aminotransferase are the products of proteolytic digestion. It is an interesting model system for studying the relationship between conformational fluctuations and catalytic activity.

The N-terminal sequence of 16 amino acid residues on 4-aminobutyrate aminotransferase was determined by automated Edman degradation. The PTH-amino acid derivatives produced in sequencing analysis indicates that the two subunits of the enzyme are homologous polypeptide chains. A tryptic peptide of 20 amino acids containing phosphopyridoxyl-lysine was isolated from the transaminase and its primary structure was determined by solid-phase technique. The determined sequence was compared with those of cytosolic and mitochondrial aspartate aminotransferase. Further investigations on the complete sequence of the enzyme as well as chrystallographic studies will provide useful information in understanding active site chemistry and structure of 4-aminobutyrate aminotransferase.

LIST OF REFERENCES

LIST OF REFERENCES

1. Van den Berg, C.J. (1970), Handbook of Neurchemistry, 3, 355-379.

2. Otsuka, M., Iversen, L.L., Hall, Z.W., and Kravitz, E.A. (1966), Proc. Nat. Acad. Sci. USA, 56, 1110-1115.

3. Krnjecic, K. and Schwarz, S. (1967), Exp. Brain. Res., 3, 320-326.

4. Roberts, E (1976), GABA in Nervous System Function, pp. 1-5, Raven Press, New York (Roberts, E., Chare, T.N. and Tower, D.B., eds.).

5. Schousboe, A., Wu, J-Y, and Roberts, E. (1973), Biochemistry, 12, 2868-2873.

6. Bloch-Tardy, M., Rolland, M.D., and Gonnard, P. (1974), Biochimie, 56, 823-832.

7. Cash, C., Maitre, M., Ciesielski, L., and Mandel, P. (1974), FEBS Letters, 47, 199-203.

8. John, R.A. and Fowler, L.J. (1976), Biochem. J., 155, 645-651.

9. Beeler, T. and Churchich, J.E. (1978), Eur. J. Biochem., 85, 365-371.

10. Maitre, M., Ciesielski, L., Cash, C. and Mandel, P. (1975), Eur. J. Biochem., 52, 157-169.

11. Yonaha, K. and Toyama, S. (1980), Arch. Biochem. Biophys., 200, 156-164.

12. Schousboe, A., Saito, K., and Wu, J-Y (1980), Brain Res. Bull., 5, 71-76.

13. Ho, P.P.K., Young, A.L. and Walters, P.C. (1975), Enzyme, 19, 244-245.

14. Buzenet, A.M., Fages, C., Bloch-Tardy, M. and Gonnard, P. (1978), Biochim. Biophys. Acta., 522, 385-399.

15. Maitre, M., Ciesielski, L., Cash, C. and Mandel, P. (1978), Biochim. Biophys. Acta., 522, 385-399.

16. Rando, R.R. and Bangerter, F.W. (1977), Biochem. Biophys. Res. Comm., 76, 1276-1281.

17. Rando, R.R. and Bangerter, F.W. (1977), J. Am. Chem. Soc., 99, 5141-5145.

18. Rando, R.R. (1977), Biochemistry, 16, 4604-4610.

19. Burnett, G., Yonaha, K., Toyama, S., Soda, K. and Walsh, C. (1980), J. Biol. Chem., 256, 428-432.

20. Tunnicliff, G., Ngo, T.T., Rojo-Ortega, J.M. and Barbeau, A. (1977), Can. J. Biochem., 55, 479-484.

21. Fowler, L.J. and John, A (1981), Biochem. J., 197, 149-154.

22. Churchich, J.E. and Moses, U. (1981), J. Biol. Chem., 256, 1101-1104.

23. Moses, U. and Churchich, J.E. (1980), Biochim. Biophys. Acta, 613, 392-400.

24. Saito, K., Schousboe, A., Wu, J-Y. and Roberts, E. (1974), Brain Res., 65, 287-296.

25. Simonsen, D.G. and Roberts, E. (1974), Biochem. Med., 10, 36-49.

26. Wu, J-Y., Moss, L.G., and Chude, O. (1978), Neurochem. Res., 3, 207-219.

27. Hyde, J.C. and Robinson, N. (1974), J. Neurochem., 23, 365-367.

28. Hyde, J.C. and Robinson, N. (1976), Histochemistry, 46, 261-268.

29. Kuriyama, K., Haber, B., Sisken, B. and Roberts, E. (1966), Proc. Nat. Acad. Sci. USA, 55, 846-852.

30. Robinson, N. and Wells, F. (1973), J. Anat., 114, 365-378.

31. Van Gelder, N.M. (1965), J. Neurochem., 12, 231-237.

32. Balazs, R., Dahl, D. and Harwood, J.R. (1966), J. Neurochem., 13, 897-905.

33. Salganicoff, L. and DeRobertis, E. (1965), J. Neurochem., 12, 287-309.

34. Van Den Berg, C.J., van Kempen, G.M.J., Schade, J.P. and Veldstra, H. (1965), J. Neurochem., 12, 863-869.

35. Waksman, A., Rubinstein, M.K., Kuriyama, K. and Roberts, E. (1968), J. Neurochem., 15, 351-357.

36. Schousboe, I., Bro, B. and Schousboe, A. (1977), Biochem. J., 162, 303-307.

37. Mishima, H., Kurihara, H., Kobayashi, K., Miyazawa, S. and Terehara, A. (1976), Tetrahedron Lett., 7, 537-540.

38. Rando, R.R. and Bangerter, F.W. 91976), J. Am. Chem. Soc., 98, 6762-6764.

39. Kwok, F. and Churchich, J.E. (1980), J. Biol. Chem., 255, 882-887.

40. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951), J. Biol. Chem., 193, 265-275.

41. Wada, H. and Snell, E.E. (1961), J. Biol. Chem., 236, 2089-2095.

42. Blaner, W.S. and Churchich, J.E. (1979), J. Biol. Chem., 254, 1794-1798.

43. Davis, B.J. (1964), Ann. N.Y. Acad. Sci., 121, 404-427.

44. Weber, K. and Osborn, M. (1969), J. Biol. Chem., 244, 4406-4412.

45. Tiselius, A., Hjerten, S. and Levin, O. (1950), Arch. Biochem. Phiphys., 65, 132-136.

46. Lee, Y.H. and Churchich, J.E. (1975), J. Biol. Chem., 250, 5604-5608.

47. Vasilev, V., Yu and Eremin, V.P. (1968), Biokhimya, 33, 1143-1149.

48. Benson, J.R. and Hare, P.E. (1975), Proc. Natl. Acad. Sci. USA, 72, 619-622.

49. Shimomura, S. and Fukui, T., (1978), Biochemistry, 17, 5359-5367.

50. Kim, D.S. and Churchich, J.E. (1981), Biochem. Biophys. Res. Comm., 99, 1333-1340.

51. Kim, D.S. and Churchich, J.E. 91982), J. Biol. Chem., 257, 10991-10995.

52. Churchich, J.E. (1982), Eur. J. Biochem., 126, 507-511.

53. Ellman, G.L. (1959), Arch. Biochem. Biophys., 82, 70-76.

54. Lamelli, U.K. (1970), Nature, 227, 680-685.

55. Manwell, C. (1977), Biochem. J., 165, 487-495.

56. Simpson, R.J., Neuberger, M.R. and Liu, T.Y., (1976), J. Biol. Chem., 251, 1936.

57. Schiltz, E. and Reinbolt, J. (1975), Eur. J. Biochem., 56, 467-481.

58. Schachman, H.K., Gropper, L., Hanlon, S., Putney, F. (1962), Arch. Biochem. Biophys., 99, 175-190.

59. Demas, J.N. and Adamson, A.W. (1971), J. Phys. Chem., 75, 2463-2466.

60. Hargrove, J.L., Gohda, E., Pitot, H., and Granner, D.K. (1982), Biochemistry, 21, 283-289.

61. Sandmeier, E. and Christen, P. (1980), J. Biol. Chem, 255, 10284-10289.

62. Careri, G., Fasella, P., and Gratton, E. (1979), Ann. Rev. Biophys. Bioeng., 8, 69-97.

63. Lakowicz, J.R. and Weber, G. (1973), Biochemistry, 12, 4171-4179.

64. Boeker, E.A., Fischer, E.H. and Snell, E.E. (1971), J. Biol. Chem., 246, 6776-6789.

65. Schachtele, K.H., Schiltz, E. and Palm, D. (1978), Eur. J. Biochem., 92, 427-435.

66. Huang, Y.Z. and Snell, E.E. (1972), J. Biol. Chem., 247, 7358-7364.

67. Schiltz, E. and Schmitt, W. (1981), FEBS Lett., 134, 57-62.

68. Fischer, E.H., Cohen, P., Fosset, M., Muir, L.W., and Soari, J.C. (1972), Metabolic Interconversion of Enzymes (Wieland, O., Helmreich, E. and Holzer, H., eds.), pp. 11-28, Springer, Berlin, Heidelberg, New York.

69. Cohen, P., Soari, C.L., and Fischer, E.H. (1973), Biochemistry, 12, 5233-5241.

70. Lerch, K. and Fischer, E.H. (1975), Biochemistry, 14, 2009-2014.

71. Kagamiyawa, J., Sakakibara, R., Wada, H., Tanase, S. and Morino, Y. (1977), J. Biochem., 82, 291-294.

72. Barra, D., Bossa, Fi., Doonan, S., Fiahmay, H.M.A., Hughes, G.J., Kakoz, K.Y., Martini, Fi., and Petruzzelli, R. 91977), FEBS Lett., 83, 241-244.

73. Kim, D.S., Moses, U. and Churchich, J.E. (1981), Eur. J. Biochem., 118, 303-308.

74. Schnackerz, K.D. and Noltmam, E.A. 91971), Biochemistry, 10, 4837-4842.

VITA

Doo Sik Kim was born on April 13, 1951, in Chun Chon City, Korea, where he received his elementary and high school education.

He entered the Yonsei University, Seoul, Korea, in March, 1969 and graduated with a Bachelor of Science degree in biochemistry in February, 1973.

He was called into the Korean Army as a chemical lieutenant in March, 1973 and served there until June, 1975.

He started his graduate study at the Yonsei University Graduate School in 1976 and obtained a Master of Science degree in biochemistry in February, 1978.

He was a research associate at Seoul National University from March, 1978 to February, 1979. He married Miss Sanghyun Chung in August, 1979.

In September of 1979, he entered Graduate School at the University of Tennessee, Knoxville and received his Ph.D. in biochemistry in March, 1983.

During the period of his doctoral research, his first daughter Nayung was born in July, 1980 at Knoxville.

In the summer of 1982, Professor Jorge E. Churchich and the author visited at the University of Wurzburg, West Germany where they worked on ^{31}P-NMR spectroscopy and primary structure of the catalytic site of 4-aminobutyrate aminotransferase.

In March of 1983, the author was appointed as a post-doctoral fellow in the laboratory of Dr. Lewis C. Cantley, Department of Biochemistry and Molecular Biology, College of Arts and Sciences, Harvard University.

He has the following publications:

Biochemical Studies on Ginseng Saponins(X): The effect of ginseng saponins on lipid metabolism. Joo, C.N., Lee, H.B., and Kim, D.S. (1977) Korean Biochem. J. 10, 71-84.

Biochemical Studies on Ginseng Saponins(XI): The effect of ginseng saponins on aldehyde dehydrogenase. Joo, C.N., Koo, J.D., Kim, D.S. and Lee, S.J. (1977) Korean Biochem. J. 10, 109-120.

Effect of Ginseng Saponins on the Absorption of Water-Insoluble Vitamins such as Vitamin A and E. Joo, C.N., Kim, D.S. and Jeon, Y.W. (1978) J. Natural Science Research Institute, Yonsei University 1, 81-86.

Biochemical Studies on Ginseng Saponins (XVI): The effect of ginseng saponin on hypercholesterolemia induced by prolonged cholesterol feeding in rabbits. Joo, C.N., Kim, D.S. and Koo, J.H. (1980) Korean Biochem. J. 13, 51-58.

4-Aminobutyrate Aminotransferase, The Reaction of Lysine Residues Connected with Enzymatic Activity. Kim, D.S. and Churchich, J.E. (1981) Biochem. Biophys. Res. Comm. 99, 1333-1340.

Gabaculine and m-Carboxyphenyl Pyridoxamine 5-Phosphate as Probes of the Catalytic Binding Sites of 4-Aminobutyrate Aminotransferase. Kim, D.S., Moses, U. and Churchich, J.E. (1981) Eur. J. Biochem. 118, 303-308.

Non-equivalent Binding Sites in 4-Aminobutyrate Aminotransferase. (Abstract) Kim, D.S., Moses, U. and Churchich, J.E. (1981) Fed. Proc. 40, 1831.

4-Aminobutyrate Aminotransferase, Reaction of P^1P^2-Bis(5'-pyridoxal) Diphosphate with Lysyl Residues Connected with Catalytic Activity. Kim, D.S. and Churchich, J.E. (1982) J. Biol. Chem. 257, 10991-10995.

Rotational Dynamics of 4-Aminobutyrate Aminotransferase. Churchich, J.E., Kim, D.S. and Schnackerz, K.D. (1983) submitted to J. Biol. Chem.

Primary Structure of the Catalytic Site of 4-Aminobutyrate Aminotransferase. (Abstract) Kim, D.S., Schiltz, E. and Churchich, J.E. (1983) Fed. Proc., in press.

Catalytic and Structural Properties of Trypsin Treated 4-Aminobutyrate Aminotransferase. Kim, D.S. and Churchich, J.E. (1983) Submitted to J. Biol. Chem.