To the Graduate Council:

I am submitting herewith a dissertation written by Francis Shu-Lai Kwok entitled "Studies on the Enzymes Pyridoxal Kinase and Pyridoxine Phosphate Oxidase." I 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

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STUDIES ON THE ENZYMES PYRIDOXAL KINASE AND PYRIDOXINE PHOSPHATE OXIDASE

A Dissertation

Presented for the

Doctor of Philosophy

Degree

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Francis Shu-Lai Kwok

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ABSTRACT

Pyridoxal kinase has been purified 2000-fold from pig brain. The enzyme preparation migrates as a single protein and activity band on analytical gel electrophoresis. The enzyme requires a divalent and a monovalent metal ion for its activity. Of the divalent metal ions studied, Zn^{+2} is the most effective in catalyzing the formation of pyridoxal-P from ATP and pyridoxal; the order of activation is $Zn^{+2} > Co^{+2} > Mn^{+2} > Mg^{+2} > Fe^{+2}$. On the other hand, the monovalent cation required for enhancing its enzymatic activity is determined to be K^{+}. Not only can pyridoxal kinase phosphorylate pyridoxal, it can also phosphorylate pyridoxine and pyridoxamine from ATP and Zn^{+2} ion. This enzyme is shown to contain one single subunit of molecular weight 60,000.

Different biogenic amines have been found in our laboratory to inhibit the pyridoxal kinase. By attaching a hydrophobic dansylated group (dansyl = 5-dimethylaminonaphthalene-1-sulfonyl) to some of the biogenic amines, the potency of inhibition is greatly enhanced. The inhibitory level of γ-aminobutyric acid is increased by 1000 fold after its modification to dansylated γ-aminobutyric acid. The biogenic amines show competitive inhhbition with respect to ATP and uncompetitive to pyridoxal.

The interactions of the substrate pyridoxal and inhibitor N-dansyl-2-oxopyrrolidine with the catalytic site have been examined by means of fluorescence spectroscopy. The increase in emission anisotropy that follows the binding of pyridoxal to the kinase is used to determine the

equilibrium constant. Pyridoxal kinase binds one molecule of substrate with K_d = 1μM at pH 6. The emission anisotropy spectrum of pyridoxal reveals that the substrate is not rigidly trapped by the protein matrix.

N-dansyl-2-oxopyrrolidine is a competitive inhibitor of pyridoxal kinase with respect to ATP at saturating concentrations of pyridoxal. It binds to the enzyme with a dissociation constant of 6μM. N-dansyl-2-oxopyrrolidine is immobilized by strong interactions with the enzyme, but it is displaced from the catalytic site by ATP. The results are consistent with the hypothesis that N-dansyl-2-oxopyrrolidine binds at the nucleotide binding site of pyridoxal kinase.

Irradiation of pyridoxal kinase in the presence of riboflavin leads to irreversible loss of catalytic activity. Riboflavin binds to the kinase with K_d = 5μM without any inhibitory effect as shown by fluorometric titrations. Singlet excited oxygen, generated by energy transfer from the lowest triplet of riboflavin to oxygen, acts as the oxidizing agent of approximately one histidine residue per mole of enzyme. The amino acid residues, such as tyrosine, tryptophan and cysteine are not photooxidized by the sensitizer bound to the enzyme. It is postulated that histidine is involved in the binding of the substrate ATP to the catalytic site of pyridoxal kinase.

Purification of pyridoxine-P oxidase has also been achieved from pig brain. The enzyme preparation is regarded as pure since it migrates as a single protein and activity band on the analytical gel electrophoresis. This enzyme catalyzes the oxidation of a primary

alcohol, a primary amine and a secondary amine. Molecular weight of the enzyme is determined to be 62,000 by sucrose density gradient centrifugation and sodium dodecyl sulfate polyacrylamide gel electrophoresis. In addition, sodium dodecyl sulfate polyacrylamide gel electrophoresis reveals that pyridoxine-P oxidase consists of two identical subunits of molecular weight 31,000 each. Pyridoxine-P oxidase, like many other oxidases, requires FMN as the cofactor for its enzymatic activities. Pyridoxine-P oxidase can be resolved from the holoenzyme to its apo- form and active holoenzyme can be reformed by addition of exogenous FMN to the apoenzyme. One sulfhydryl group per molecule of enzyme is titrated by DTNB on pyridoxine-P oxidase without any loss of enzymatic activity. Attachment of fluorescent probe, such as iodoacetamide fluorescein, on pyridoxine-P oxidase does not affect its catalytic activity.

Complex formation between pyridoxal kinase and pyridoxine-P oxidase is detected; the results of the energy transfer experiment reveal that the two enzymes are approximately 35Å apart in solution. Increase of polarization of fluorescence from 0.2 to 0.25 further confirm the formation of the protein-protein complex when pyridoxine-P oxidase is mixed with equal molar concentration of pyridoxal kinase. Further evidence supporting protein-protein interaction was obtained by Sephadex G-100 chromatography, sucrose density gradient centrifugation, and transient kinetic analysis of the coupled assay system. The kinetic analysis used pyridoxine as the substrate for pyridoxal kinase and then pyridoxine-P oxidase continued the oxidation reaction to form pyridoxal-P.

Reduction of the transient time for the coupled assay system provides evidence for the possibility of interaction between the two enzymes. A functional role played by the enzyme-enzyme complex in the regulation of accumulation of pyridoxal-P is discussed.

TABLE OF CONTENTS

CHAPTER PAGE

CHAPTER PAGE

LIST OF TABLES

LIST OF FIGURES

FIGURE PAGE

FIGURE PAGE

CHAPTER I

PYRIDOXAL KINASE

A. INTRODUCTION, PURIFICATION AND CHARACTERIZATION

Pyridoxal kinase is an enzyme of utmost biological interest since it catalyzes the formation of the active species of vitamin B_6 from its vitamers. The formation of pyridoxal-P catalyzed by pyridoxal kinase from pyridoxal and ATP is essential for many transamination, decarboxylation and racemization reactions. Besides the conversion of pyridoxal to pyridoxal-P, this enzyme can also phosphorylate pyridoxine and pyridoxamine from ATP. The phosphorylation of the three vitamers by pyridoxal kinase (ATP: pyridoxal 5'-phosphotransferase EC 2·7·1·35) requires the presence of a divalent cation and a monovalent cation. The most effective divalent cation is Zn^{+2} and the order of activation is $Zn^{+2} > Co^{+2} > Mn^{+2} > Mg^{+2} > Fe^{+2}$. The monovalent cation is K^{+}.

In 1944, Gunsalus, Bellamy and Umbreit discovered that the active form of vitamin B_6 is pyridoxal-P (1), which is now known to serve as cofactor in the enzymatic transformations of all amino acids (2,3,4). Pyridoxal kinase is the key enzyme responsible for the phosphorylation of the vitamin B_6 and has been detected in brain (2), liver (5), Streptococcus faecalis (1,6), Escherichia coli (7) and yeast (7). A detailed investigation on the distribution of pyridoxal kinase in rat and bovine tissues has been conducted by McCormick et al. (8) and brain has been found to be a rich source of the enzyme. Purifications of this

enzyme from different sources have been attempted in different laboratories (8,9), however, only partial purifications of pyridoxal kinase have been achieved.

Many pyridoxal analogs have been reported to be inhibitors of pyridoxal kinase (10,11) and these analogs are primarily oximes, azines and hydrazones of pyridoxal. Biogenic amines have also been reported to inhibit pyridoxal kinase (12), but no information has been reported on the interaction of biogenic amines with the purified kinase.

Pyridoxal kinase has also been reported as a key enzyme related to the transport of vitamin B_6 in Salmonella Typhimurium LT2 cells (13). The uptake of pyridoxine by facilitated diffusion through the cell membrane is followed by trapping by pyridoxal kinase. Other studies of transport of vitamin B_6 concerning pyridoxal kinase in the brain have been reported (14,15).

The physiological significance of pyridoxal kinase is not fully known. However, during a comparative study of pyridoxal kinases from different organisms (8), brain tissue has been found to be the richest source of this enzyme. This finding is of particular interest because of the intimate relationship of vitamin B_6 to brain metabolism as evidenced by the occurrence of convulsive seizures during vitamine B_6 deficiency, whether induced by restricted intake of the vitamin (16,17,18), by administration of analogs of the vitamin (17,19) or by administration of carbonyl reagents such as isonicotinyl hydrazide and related compounds (20,21). Other <u>in vivo</u> studies in rabbits have demonstrated that when the concentrations of various biogenic amines were increased by compounds which inhibit their metabolism, the activity of pyridoxal

kinase in the brain was reduced (22). Furthermore, chronic treatment with levodopa alters the activity of pyridoxal kinase in rat brain (23) and also elevates the concentration of pyridoxal-P in plasma of Parkinsonian patients (24).

B. PURIFICATION OF PYRIDOXAL KINASE

Materials

Fresh pig brain was acquired from East Tennessee Packing Company, Knoxville, Tennessee. CM-Sephadex C-50, Sephadex G-100, DEAE-Sephadex A-25 and Sepharose AH were purchased from Pharmacia Fine Chemicals. Hydroxyapatite gel was prepared according to the method of Tiselius (25).

Pyridoxal, pyridoxine, pyridoxamine, pyridoxal-P, glutathione and 4-deoxypyridoxine were purchased from Sigma Chemical Company. Potassium phosphate (monobasic and dibasic), zinc chloride and ammonium sulfate were from Fisher. All other chemicals used in this research were of reagent grade.

Methods for Assay of Pyridoxal Kinase

There are five methods for determining the enzymatic activity of pyridoxal kinase. All five methods of assay used the same standard assay solution which contained 1×10^{-4}M pyridoxal, 2.5×10^{-4}M ATP and 5×10^{-6}M $ZnCl_2$ in a total volume of 1ml in 0.07M potassium phosphate (pH 6).

The most frequently used method is the spectrophotometric assay by monitoring the formation of pyridoxal-P which has an absorption

maximum at the wavelength of 388nm (ε_{388} = 4900 $cm^{-1}M^{-1}$ at pH 7). The enzymatic reaction can be followed in the Aminco DW-2 spectrophotometer at 25°C. Optical density of 0.1 as full scale is used for this assay.

The second method of assay is by utilizing the reaction between pyridoxal-P and phenylhydrazine. The product of the reaction gives an absorption maximum at 410nm (ε_{410nm} = 23,000). The quantity of pyridoxal-P formed in the assay reaction mixture can be determined by a standard curve. This method is usually applied in our research for assaying the crude homogenate of pig brain because spectrophotometric methods cannot be applied due to the light scattering effect of the turbid assay solution. In the presence of enzyme, the assay mixture was incubated at 25°C or 37°C in a water-bath for 30 minutes. After the incubation, 0.1ml of 100% trichloroacetic acid was added to the assay mixture and the assay mixture was subjected to centrifugation at 6000 rpm in a desk-top centrifuge for 10 minutes. One ml of the supernatant was removed to another test-tube and 0.1ml of 2N phenylhydrazine in 10N H_2SO_4 was added. The mixture was read in the spectrophotometer at 410nm after 3 minutes.

The third method of assaying pyridoxal kinase is by monitoring the formation of ADP which can be measured by using a coupled assay of pyruvate kinase and lactate dehydrogenase in the presence of phosphoenolpyruvate and NADH. One x 10^{-4}M phosphoenolpyruvate, 5 x 10^{-6}M $MgCl_2$, 1 x 10^{-4}M NADH, 50µg of pyruvate kinase and 50µg of lactate dehydrogenase were added to the standard assay mixture in the presence of pyridoxal kinase, the reaction can be followed in the Aminco DW-2

spectrophotometer at 340nm at 25°C. The extinction coefficient used for NADH at 340nm is $6220cm^{-1}M^{-1}$.

The fourth method is by using radioactive ^{32}P labelled at the γ position of ATP. The enzymatic reaction results in the transfer of the radioactive phosphate from ATP to pyridoxal, pyridoxine or pyridoxamine. The separation of the radioactive ATP and pyridoxal-P can be accomplished by using a DEAE Sephadex A-25 column chromatography. DEAE Sephadex A-25 column was packed in a Pasteur pipette and was equilibrated by 0.07M potassium phosphate (pH 6). The standard assay mixture, in the presence of enzyme, pyridoxamine instead of pyridoxal and $\gamma^{32}P$ ATP, was incubated in a water-bath at 37°C or 25°C for 30 minutes. The final reaction mixture consisting of radioactive ATP and radioactive pyridoxal-P was applied to the DEAE column and the column was subsequently washed by 2ml of 0.07M potassium phosphate buffer (pH 6). After washing, the column was eluted with 2ml of 0.07M potassium phosphate buffer (pH 2.5) and 1ml fractions were collected from the column. The fractions collected mainly contained radioactive pyridoxal-P and were quantitated in a scintillation counter. This method is desighed for assaying the substrate pyridoxamine for pyridoxal kinase because spectrophotometric means for the assay of pyridoxamine are not feasible.

The fifth method for assaying pyridoxal kinase is to use the coupled assay with pyridoxine-P oxidase. The assay is specifically designed for the substrate pyridoxine. In the presence of enzyme, the assay mixture was incubated at 37°C for 1/2 hour and subsequently, the reaction was stopped by addition of $5 \times 10^{-5}M$ dansylated γ-aminobutyric acid. Then the pH was re-adjusted to pH 8 and $5 \times 10^{-6}M$ FMN and 100μg

of pyridoxine-P oxidase were added. The resulting mixture was left at 37°C for 4 hours of incubation and the final reaction mixture was read by a spectrophotometer at 388nm for change of absorbance.

Protein concentrations were determined by the method of Lowry (26) and also at 280nm spectrophotometrically using an extinction coefficient of $60,000M^{-1}cm^{-1}$.

Synthesis of 4-Deoxypyridoxyl-Sepharose

Four-deoxy-5-pyridoxaldehyde was prepared according to the method of Sattsangi and Argoudelis (27) by the oxidation of 4-deoxypyridoxine with manganese dioxide. A mixture of 16g of manganese dioxide and 4g of 4-deoxypyridoxine in 40ml of chloroform was stirred at room temperature for 24 hours in the dark. The reaction mixture was diluted with 80ml of chloroform and filtered with suction through a layer of Whatman No. 3 filter paper. The contents of the funnel were washed with five 200ml portions of chloroform. The combined filtrate and washings were evaporated under reduced pressure to an oily residue to which was added 80ml of petroleum ether (b. pt. 40-50); it was then warmed and filtered. The filtrate was treated with a small amount of activated charcoal, filtered again, and kept in a refrigerator overnight until crystalline 4-deoxypyridoxaldehyde was obtained.

The final product was reacted with phenylhydrazine to demonstrate that an aldehyde group has been formed. Four-deoxypyridoxaldehyde is an inhibitor of pyridoxal kinase at a concentration of $5 \times 10^{-5}M$.

Sepharose, derivatized with N'-(ω'aminohexyl)-4-deoxypyridoxyl, was prepared by reacting the "spacer group" (ω-aminohexyl) of the

Sepharose-AH (Pharmacia) with 4-deoxypyridoxaldehyde (2,4-dimethyl-5-formyl-3-hydroxypyridine) at pH 7. Six g of Sepharose AH were suspended in 50ml of water, brought to pH 7 by addition of NaOH, mixed with 1g of 4-deoxy-5-pyridoxaldehyde and allowed to react at 25°C in the dark for 12 hours. The resulting Schiff base was reduced by addition of 20mg of solid sodium borohydride at 4°C. After reduction, the derivatized Sepharose was washed several times with water. Then the gel was ready for purification after equilibration with buffer.

Purification Procedure

The enzyme, pyridoxal kinase, has been partially purified in different laboratories (8,9) but no homogeneous preparation has been reported in the literature. The latest method of purification developed by Neary (9) from bovine brain was not applicable to the isolation of this enzyme from pig brain because of two reasons. Firstly, DEAE-cellulose and ADP-agarose both could not retain pyridoxal kinase from pig brain and no purification could be achieved from these two column chromatography methods. Secondly, Tris-HCl buffer which was used in the purification of pyridoxal kinase from bovine brain, would react with pyridoxal-5-P to form the Schiff base. The formation of the Schiff base which absorbs at 410nm maximally, would interfere with the spectrophotometric assay of the enzyme. Therefore, a new procedure for the purification of pyridoxal kinase from pig brain was developed in our laboratory.

Pig brains were obtained from East Tennessee Packing Company. The brains were placed in ice as quickly as possible after slaughter, and

preparation was begun within one hour. All preparation procedures were carried out at 4°C in a cold room.

A Waring Blendor was used to prepare a 25% (w/v) homogenate in a solution of 0.075M potassium phosphate (pH 6.8). The homogenate was filtered through several layers of cheese cloth. The filtrate was centrifuged at 10,000 x g for 30 minutes and the precipitate was discarded. The supernatant was then treated with $(NH_4)_2SO_4$. The precipitate obtained at 40-60% saturation was dissolved in 0.01M potassium phosphate (pH 6.8) containing 0.1mM glutathione, 0.02mM $ZnCl_2$ and 0.05mM pyridoxal (Buffer I). It was then dialysed against buffer I several times to remove all the ammonium sulfate from the enzyme and applied to a column (35 x 2.6cm) of CM-Sephadex equilibrated with the same buffer. The column was eluted with buffer I; the enzyme was not retained by CM-Sephadex, whereas the red contaminating proteins remained attached to the supporting gel. The active fractions were combined and applied to a column (25 x 2.6cm) of hydroxyapatite equilibrated with buffer I. The enzyme was eluted by using a linear gradient made with equilibration buffer (250ml) and the same volume of 0.3M potassium phosphate (pH 6.8) containing 0.05mM pyridoxal, 0.02mM $ZnCl_2$ and 0.1mM glutathione (buffer II). The active fractions were combined and applied to a column (100 x 2.6cm) of Sephadex G-100, previously equilibrated with the same buffer. The enzyme was then chromatographed on a column (14 x 0.9cm) of 4-deoxypyridoxyl-Sepharose equilibrated with buffer I. The enzyme was retained by the derivatized Sepharose and eluted by increasing concentrations of potassium chloride from 0.1 to

0.2M. The enzyme is eluted at a concentration of potassium chloride of 0.15M. The results from one such purification are shown in Table I. The purified enzyme was kept in 75mM potassium phosphate (pH 6.8) containing 0.05mM pyridoxal, 0.02mM $ZnCl_2$ and 0.1mM glutathione at -20°C. Under these conditions, the enzyme remains stable for at least two months without appreciable loss in activity. The chromatography on derivatized sepharose, which is the last step in the purification of pyridoxal kinase, permits the separation of several contaminating proteins. The fraction of pyridoxal kinase obtained after 4-deoxypyridoxyl-sepharose chromatography was found to be homogeneous, as only one protein band with enzymatic activity was detected by slicing an unstained gel into 1mm sections, macerating the slices in a solution containing 0.5ml 0.07M potassium phosphate, 0.05mM pyridoxal, 0.02mM $ZnCl_2$, 0.1mM glutathione, 0.1mM ATP, allowing the suspension to stand overnight and assaying each sample by the phenylhydrazine method.

C. CHARACTERIZATION OF THE ENZYME

The purified enzyme was tested for homogeneity by polyacrylamide gel electrophoresis. Activity of pyridoxal kinase from the unstained gel was assayed.

Materials

Photo flow 200 was from Eastman Kodak. Acrylamide, N,N'methylene bisacrylamide, N,N,N',N' tetrametnylene diamene, and naphthol blue black was from Eastman Organic. Acetic acid and ammonium persulfate were from Fisher.

TABLE I

PURIFICATION OF PYRIDOXAL KINASE FROM PIG BRAIN

The purification of pyridoxal kinase was made from 5 Kg of pig brain tissue, wet weight

Treatment	Volume (ml)	Protein (mg)	Protein (mg/ml)	Specific* Activity (Units/mg at 37°C)	Total Activity Units
Homogenate	7000	117,600	16.8	0.039	4586
Dialyzed, 40-60% $(NH_4)_2SO_4$ Fraction	700	22,050	31.5	.1	2205
CM-Sephadex Fraction	700	17,782	25.4	.12	2133
Hydroxyapatite Fraction	200	1,600	8	1	1600
Sephadex G100 Fraction	50	55	1.1	10	550
4-Deoxypyridoxyl-Sepharose	8	6.4	0.8	71.3	456

*A Unit of Activity is defined as that amount of protein which catalyzes the formation of 1 nanomole of pyridoxal-5-phosphate per minute at 37°C.

Methods

The homogeneity of pyridoxal kinase was determined by polyacrylamide gel electrophoresis in a water-cooled Buchler polyanalyst (Buchler Instruments) by the method of Davis (52). The glass tubes were rinsed with photo-flow and allowed to dry. Into each tube was placed 100µl of sucrose. Then 1ml of 7% separating gel was pipetted into each tube. Water was applied to the top of the gel solution before they were set, and gelling time took 30 minutes or less. The water was removed and 100µl of stacking gel was applied to each tube and water was then layered on the top. The gel was photopolymerized in 30 minutes by using a desk lamp (100 watts). After polymerization, the water was removed and the gels placed in the holders of the upper chamber. The samples, approximately 20µg of protein and 10% sucrose, were applied to the top of the gels. Both chambers were filled with 0.05M Tris-glycine buffer (pH 8.3) and the electrophoresis of the samples were run for 1 hour at three milliamperes per tube. After the completion of a run, the gels were removed from the tubes and stained for protein or assayed for activity.

For protein, the fixative stain was naphthol blue black (1g in 100ml of 7% acetic acid). The gel was incubated for 15 minutes in the staining solution and then destained by dialysis overnight against four liters of 7% acetic acid. The protein appears as a blue band on the clear gel. There is no activity stain of activity for pyridoxal kinase. Therefore, the unstained gel was sliced into 1mm

sections, macerating slices in 0.5ml 0.07M potassium phosphate, that contained 0.05mM pyridoxal, 0.02mM $ZnCl_2$, 0.1mM glutathione, 0.1mM ATP, allowing the suspension to stand overnight and assaying each sample by the phenylhydrazine method.

Sucrose density gradient centrifugation was used to determine the purity and molecular weight of the enzyme according to the method of Martin and Ames (28). Sucrose density gradient was made by using the gradient marker (Buchler) with two adjacent chambers of 2.3ml each in volume. Twenty per cent (wieght per volume) of cold sucrose (0.584M) in 0.05M Tris-HCl buffer at pH 7.5 was placed in the mixing chamber and 5% (weight per volume) of cold sucrose (0.146M) in 0.05M Tris-HCl buffer at pH 7.5 was placed in the adjacent chamber. The stirrer was operated so that homogeneous stirring would be achieved. The tip of the outflow tube was placed at the top of a cellulose acetate centrifugation tube (Beckman). The gradient was stored in the cold room at 4°C for 4 to 18 hours before use. Protein of 0.1ml was layered on the gradient. A sharp interference between the sucrose and protein solution was always observed and this interference remained distinct for several minutes required to load the centrifuge tubes into the rotor buckets of the SW-39 rotor from Beckman. Spinco Model D centrifuge (Beckman Instrument Inc., Spinco Division, Palo Alta, California) was used to perform this experiment. The gradient was run for 18 hours at 34,000 rpm and the sucrose gradient was fractionated by 10 drops per test-tube.

Each tube was assayed for pyridoxal kinase activity and also the activity of the standard protein for the molecular weight determination.

The molecular weight of pyridoxal kinase was calculated from the equation below by using standard enzymes of known sedimentation coefficient and molecular weight.

$$\frac{S_1}{S_2} = \frac{MW_1}{MW_2}^{2/3} \qquad (1)$$

In equation (1), S is the sedimentation coefficient and MW is the molecular weight of macromolecules. The equation is applicable to most globular proteins which are roughly spherical in shape and have the same partial specific volume $\bar{v}$. Standard enzymes used in this experiment were lysozyme (MW = 17,200), lactate dehydrogenase (MW = 142,000) and catalase (MW = 240,000).

Results

The results of the polyacrylamide gel electrophoresis are seen in Figure 1 which is a photograph of gels containing pyridoxal kinase stained for protein. The gel showed one single band when stained by this technique, indicating that the protein is homogeneous.

The results of sucrose density gradient centrifugation are seen in Figure 2, which shows the molecular weight of pyridoxal

Figure 1. Polyacrylamide gel of pyridoxal kinase

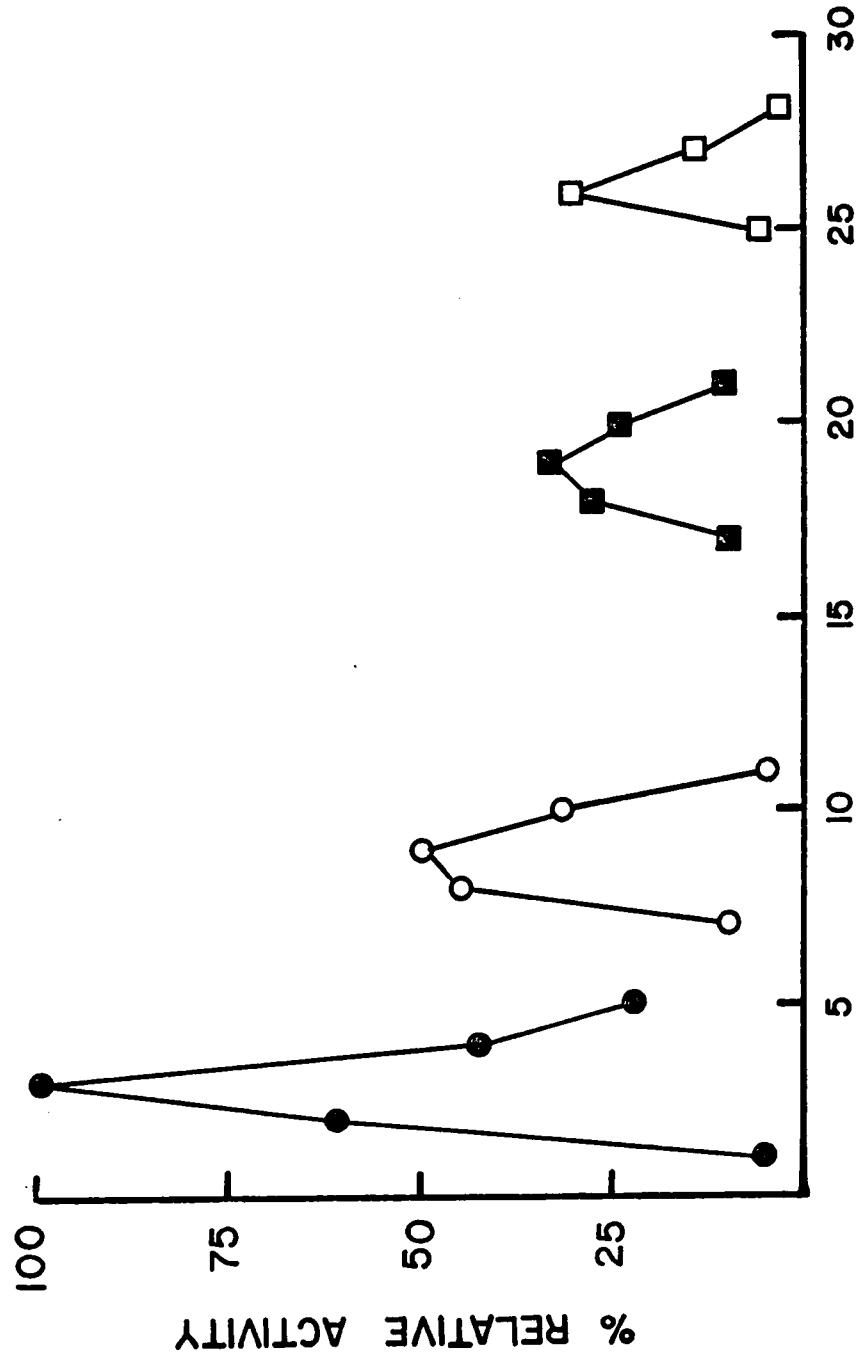

Figure 2. Sucrose density gradient centrifugation of pyridoxal kinase for molecular weight determination. Proteins of standard molecular weights used were [●] catalase, [O] lactate dehydrogenase and [□] lysozyme. [■] Pyridoxal kinase was calculated to have a molecular weight of 60,000.

kinase when compared with the molecular weight of some standard proteins. The sample of enzyme showed moved as a single band of protein and enzymatic activity of pyridoxal was detected from the protein band. As a result, the protein was homogeneously purified. The protein of pyridoxal kinase was determined to have a molecular weight of 60,000 as the result of this technique.

Discussion

The enzyme purified from pig brain appears to be homogeneous as determined by polyacrylamide gel electrophoresis and sucrose density gradient centrifugation.

D. SUBSTRATE SPECIFICITY OF THE ENZYME

Materials

Pyridoxal, pyridoxine, pyridoxamine and ATP were purchased from Sigma Chemical Company. γ labelled ^{32}P was purchased from Amersham. DEAE-Sephadex A-25 was purchased from Pharmacia Fine Chemicals. Potassium phosphate (monobasic and dibasic and $ZnCl_2$ were from Fisher.

Method

Pyridoxal was assayed according to the spectrophotometric method described previously (p. 3).

Pyridoxine was assayed by the method of coupled assay as described (p. 5). Pyridoxamine was assayed by the method using radioactive γ-labelled ^{32}P ATP for pyridoxal kinase as described on page 4.

The kinetic parameters were determined by the Michaelis-Menton equation

$$\frac{v}{V_{max}} = \frac{[S]}{K_m + [S]}$$

where v is the velocity of the enzymatic reaction. V_{max} is the maximum velocity of the enzymatic reaction. K_m is the concentration of the substrate at half of the maximum velocity. [S] is the concentration of the substrate.

Results

Pyridoxal kinase was tested for its substrate specificity. The results obtained were shown in Table II. In Table II, the K_m and V_{max} for the three substrates were determined by different assaying techniques.

Discussion

Pyridoxal kinase from pig brain can utilize the same substrates as pyridoxal kinase from different sources although K_m and V_{max} values may vary from different sources (8,9,10). The K_m and V_{max} determined previously in the literature (9) were obtained from partially purified preparations, but the K_m and V_{max} obtained here in these experiments were regarded as more significant results because it was obtained from purified pyridoxal kinase from pig brain.

TABLE II

SUBSTRATES AND INHIBITORS OF PYRIDOXAL KINASE

Compound	K_M or K_I (M)	Relative V_{max}[1]	Type of Inhibition
Pyridoxal	2.5×10^{-5}	100	
Pyridoxine	1.7×10^{-5}	46	
Pyridoxamine	1.0×10^{-5}	86	
ATP	1.2×10^{-5}		
Pyridoxaloxime	2×10^{-6}		Competitive [2]
4-deoxypyridoxine	1×10^{-5}		Competitive [2]
DANS-4-aminobutyrate	2×10^{-6}		Competitive [3]

1 V_{max} values are given relative to pyridoxal as substrate

2 Competitive with respect to pyridoxal as variable substrate

3 Competitive with respect to ATP as variable substrate

E. INHIBITORS OF PYRIDOXAL KINASE

Many pyridoxal analogs have been reported as inhibitors of pyridoxal kinase (10,11). All of these pyridoxal analogs showed competitive inhibition with respect to the substrate pyridoxal. Pyridoxine-P was found to be an inhibitor of pyridoxal kinase.

Some biogenic amines have been reported as inhibitors of pyridoxal kinase (12). More biogenic amines have been found in our laboratory to be potent inhibitors of the enzyme. The mechanism of inhibition has been studied. An increase of potency of the inhibitors by chemical modifications has been achieved.

Materials

Pyridoxine-P was prepared by reduction of pyridoxal-P by sodium borohydride and purified by crystallization. Pyridoxal, ATP, tryptamine, tyramine, γ-aminobutyric acid, histamine, DNS-tryptamine, and DNS-γ-aminobutyric acid were purchased from Sigma Chemical Company.

Method

The enzyme was assayed according to the conditions established previously on page 3. The experiment was performed in an Aminco DW-2 spectrophotometer. Initial velocity data were fitted by a least-square method using the Lineweaver-Burk transformation of equation (2)

$$v = V_{max}[S] \quad K_m + [S] \qquad (2)$$

where [S] represents the concentration of the varied substrate, K_m the Michaelis constant and V_{max} the maximum velocity.

Inhibition data corresponding to linear competitive or linear noncompetitive inhibition were fitted to the appropriate forms of equation (2).

Results

It was shown previously (10) with partially purified preparations of pyridoxal kinase that pyridoxaloximes are powerful inhibitors of the enzyme. This is also true of the purified preparation of pig brain which is inhibited by pyridoxaloxime with a K_I of 2×10^{-6}M. Several pyridoxal analogs, tested as inhibitors of the purified kinase, yielded the inhibition constants and inhibition patterns recorded in Table II. Pyridoxaloxime and 4-deoxypyridoxine inhibit the enzyme by competing with the substrate pyridoxal. Another significant result of these inhibition studies was the finding that among several dansylated derivatives of amines tested (Table III), DANS-aminobutyrate was a powerful reversible inhibitor of the kinase. The inhibitory effect exerted by DANS-aminobutyrate was not reversed by addition of potassium phosphate at concentrations greater than 70mM, indicating that the inhibitor did not compete with the monovalent potassium ions for the same binding site on the enzyme. Moreover, initial velocity patterns observed with ATP as the variable substrate and the dansylated derivative as the changing, fixed inhibitor, revealed that DANS-4-aminobutyrate was a competitive inhibitor with respect to ATP. DANS-4-aminobutyrate, which has no inhibitory effect on pyridoxine-5-P oxidase, can be used as a specific inhibitor of the kinase in reaction mixtures containing both enzymes. Besides the dansylated derivatives of amines, biogenic amines,

TABLE III

INHIBITION OF PYRIDOXAL KINASE BY BIOGENIC AMINES AND THEIR DANSYLATED DERIVATIVES

Reagent	Dissociation Constant (1) (K_D, μM)	Inhibition Constant (2) (K_I, μM)	Type of Inhibition
Kynuramine		50	Competitive
DNS-Kynuramine	6	8	Competitive
Tryptamine		100	Competitive
DNS-Tryptamine	5	4	Competitive
DNS-Tryptamine + Pyridoxal	5.2		
4-aminobutyrate		2,000	
DNS-4-aminobutyrate	1.2	1	Competitive
Histamine		400	Competitive

(1) Determined by fluorometric titrations.

(2) Determined by enzymatic assays at constant concentration of pyridoxal (0.1mM) and varying concentrations of ATP.

such as tyramine, tryptamine, histamine and γ-aminobutyric, also exerted an inhibitory effect on the enzyme with lower potency.

Discussion

Pyridoxal analogs showed competitive inhibition with respect to the substrate pyridoxal. This means that these analogs still resemble the structure of pyridoxal and compete for the same binding site of pyridoxal. Biogenic amines and their dansylated derivatives inhibit pyridoxal kinase differently by competing for the ATP binding site of the enzyme as shown by inhibition data. Most of the biogenic amines are products of the decarboxylation of amino acids and most decarboxylases require pyridoxal-P as the cofactor for its enzymatic action. The concentration of γ-aminobutyric acid in the brain is around 2×10^{-3}M and at this concentration, γ-aminobutyric acid is able to inhibit pyridoxal kinase. Therefore, the inhibitory effect of γ-aminobutyric acid on pyridoxal kinase may serve as a regulatory mechanism for the production of pyridoxal phosphate in in vivo conditions in the brain.

CHAPTER II

BINDING STUDIES OF SUBSTRATE AND INHIBITOR TO PYRIDOXAL KINASE

A. BINDING OF THE SUBSTRATE PYRIDOXAL

Materials

Pyridoxal was purchased from Sigma Chemical Company. Potassium phosphates (monobasic and dibasic) were purchased from Fisher.

Methods

Absorption spectra were measured by a Cary 15 spectrophotometer. Fluorescence emission spectra were obtained with the use of a spectrofluorometer designed in our laboratory. Exciting light was from a 200 watt Mercury-Xenon lamp (Hanovia) and was passed through a 500nm grating monochromator (Bausch and Lomb) blazed at 350nm and with dispersion of 33nm/mm and focused through a quartz lens onto a 1cm path length cell. The fluorescence was monitored through an identical monochromator to an EMI 62565 end window photomultiplier tube. Photocurrent from the tube was amplified by a Pacific Instruments Model 15 photometer. Spectra were recorded on a Hewlett-Packard 4075-A X-Y recorder. Calibration of exciting light source and spectral intensity of the photomultiplier tube have been previously described (29).

Polarization of fluorescence was measured in an instrument similar to that described by Weber (59). Illumination was provided by a Xenon-Hp lamp (150 w) with wavelengths selected by a quartz prism monochromator. The band width for excitation was 5nm. Fluorescence polarized light

was passed through a C-S-3-72 Corning glass filter. The detector system consisted of an EMI 9502B photomultiplier and a digital voltmeter. The degree of polarization of fluorescence was measured with a precision of ±0.02.

Fluorescence quantum yield measurements were made according to the method of Parker and Rees (30), using NADH in a solution of 0.1M potassium phosphate (pH 7.5) as a standard of known quantum yield (Q = 0.03). The dielectric constant of water-dioxane mixtures were obtained from the literature (31).

Results

The interactions between pyridoxal kinase and the substrate pyridoxal were studied by means of absorption and fluorescence spectroscopy. Figure 3 shows the absorption and fluorescence characteristics of pyridoxal (20μM) in the presence of pyridoxal kinase (200μM) in 70mM potassium phosphate (pH 6) at 25°C. Under this set of experimental conditions, fluorescence properties, i.e. band position and quantum yield, of bound pyridoxal are indistinguishable from those of free substrate. However, the emission anisotropy of bound pyridoxal is different from that of free pyridoxal as revealed by emission anisotropy spectra as recorded in Figure 3. Although there is a significant increase in the emission anisotropy upon binding of the pyridoxal to the enzyme, the anisotropy values attained in the presence of excess enzyme are still lower than the anisotropy values of pyridoxal in glycerol at 25°C.

The change in emission anisotropy associated with the interaction of the substrate pyridoxal with the kinase was used to determine the

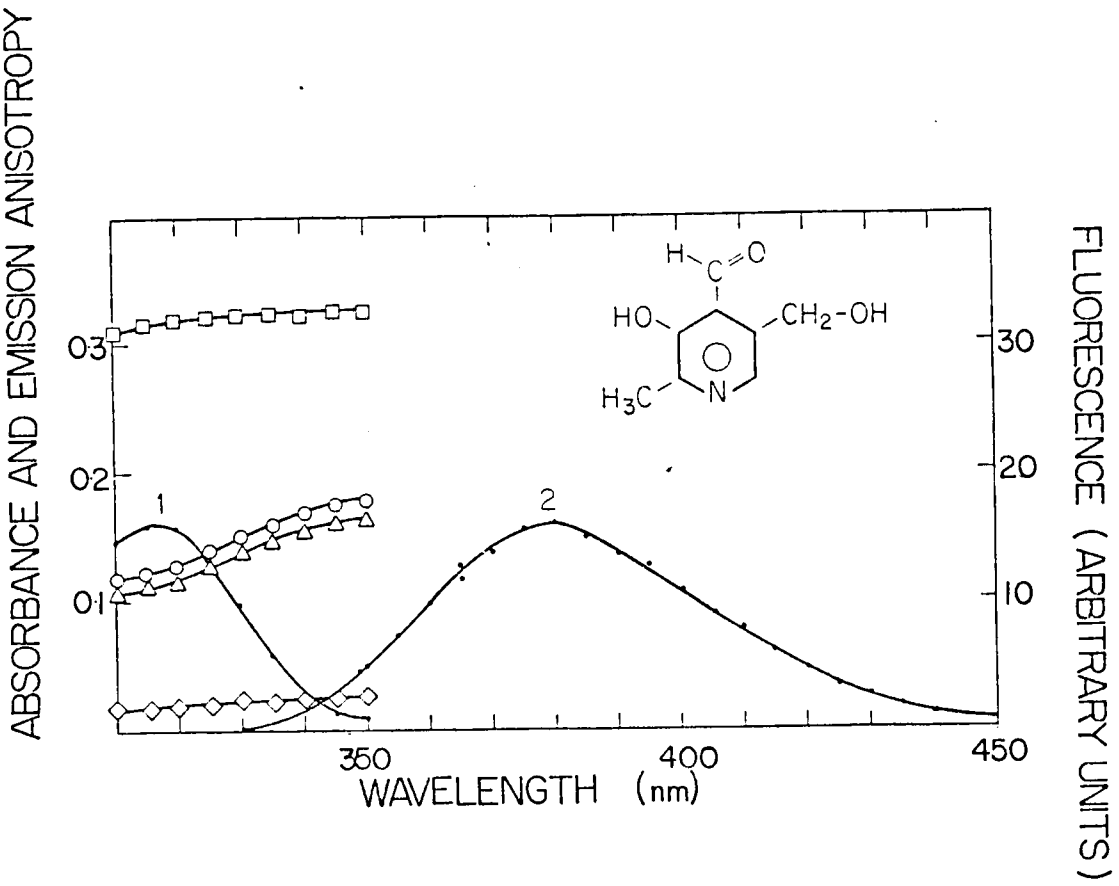

Figure 3. Absorption (1), emission (2) and emission anisotropy (△) spectra of pyridoxal (20μM) in the presence of pyridoxal kinase (200μM) in 70mM potassium phosphate (pH 6) at 25°C. Effect of addition of ADP (1mM) on the emission anisotropy of bound pyridoxal (O). Emission anisotropy spectra of pyridoxal (20μM) in glycerol (□) and water (◇) at 25°C.

stoichiometry of binding. To this end, the emission anisotropy ($\overline{A}$) of samples containing fixed concentrations of enzyme (15μM) and varying concentrations of pyridoxal was measured at constant temperature (25°C) using an excitation wavelength of 320nm.

The results of the fluorometric titrations are included in Figure 4. Since the emission anisotropy values ($\overline{A}$) obtained at varying concentrations of pyridoxal reveal the contribution of both free and bound pyridoxal to the emission anisotropy of the system, it is possible to calculate the fraction of pyridoxal bound to the enzyme (α) by using equation (3)

$$\alpha = \frac{\overline{A} - A_F}{A_B - A_F} \qquad (3)$$

where A_F and A_B are the emission anisotropies of free and bound pyridoxal, respectively, upon excitation with light of 320nm. In addition, equation (4) permits the determination of $\overline{v}$, the fractional saturation of the protein

$$\overline{V} = \alpha\frac{[L_o]}{[P_o]} \qquad (4)$$

where $[L_o]$ and $[P_o]$ are ligand (pyridoxal) and protein concentrations, respectively.

An analysis of the results included in Figure 4 by plotting $\overline{v}/[L]$ against $\overline{v}$ yielded a dissociation constant of 11μM for one binding site (n = 1) per molecule of enzyme.

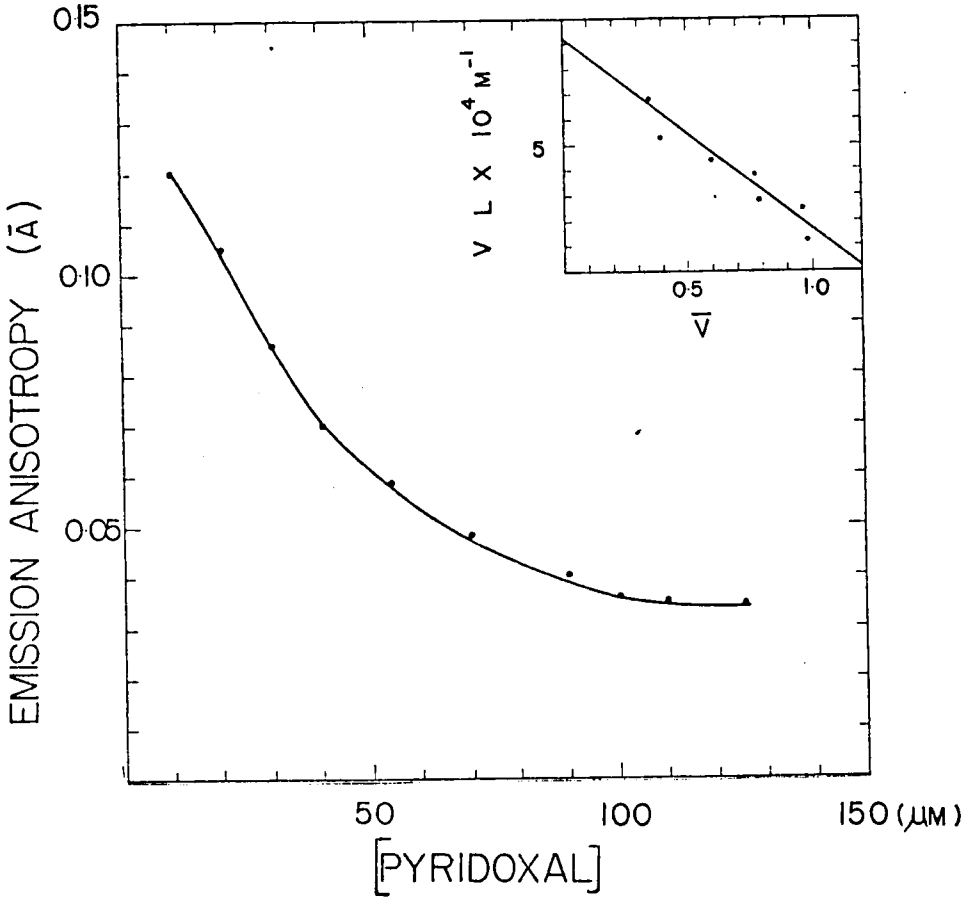

Figure 4. Changes in emission anisotropy ($\bar{A}$) upon addition of pyridoxal to a fixed concentration of enzyme (15μM) in 70mM potassium phosphate (pH 6) at 25°C. Insert: plot of $\bar{v}/[L]$ vs. $\bar{v}$. A dissociation constant of 11μM for one binding site per molecule of enzyme is obtained.

B. BINDING OF A FLUORESCENT INHIBITOR

Materials

N-dansyl-2-oxopyrrolidine and ATP were purchased from Sigma Chemical Company.

Methods

The equilibria of ligand binding to large molecules are measured in terms of dissociation constant Kd

$$Kd = \frac{[E][L]}{[EL]}$$

where [E], [L] and [EL] are the molar concentrations of free binding sites, free ligand and ligand-occupied binding sites respectively.

The dissociation constant Kd can also be represented as

$$Kd = \frac{([E^\circ]-[EL])[L]}{[EL]}$$

where [E°] is the total site concentration.

Then, in the fluorometric titration of N-dansyl-2-oxopyrrolidine to pyridoxal kinase, [E°] can be measured as the F_M which is the maximum enhancement of fluorescence when all the binding sites are completely occupied by the ligands. [EL] can also be measured as F which is the fluorescence enhancement for a certain concentration of ligand bound to the enzyme.

$$Kd = \frac{(F_M - F)[L]}{(F)}$$

$$Kd/[L] = \frac{F_M}{F} - 1$$

$$\frac{F_M}{F} = Kd/[L] + 1 \qquad (5)$$

Fluorescence measurements were conducted in the instrument previously described on page 20.

Results

The binding of N-dansyl-2-oxopyrrolidine to the enzyme is easily detected by fluorescence spectroscopy. At a protein concentration of 50μM and N-dansyl-2-oxopyrrolidine concentration of 30μM, the binding of N-dansyl-2-oxopyrrolidine to the enzyme brings about a substantial increase in fluorescence emitted at 480nm (excitation 360nm). The fluorescence quantum yield of bound N-dansyl-2-oxopyrrolidine is 50 fold enhanced when compared to the free ligand at pH 6. In addition, N-dansyl-2-oxopyrrolidine bound to the enzyme displays the following properties: Firstly, the band position of the emission spectrum is shifted toward shorter wavelengths and the magnitude of blue shift is approximately 35 nm; and secondly, the emission anisotropy spectrum fluctuates between 0.16 and 0.20 when the excitation wavelength is varied from 310 to 380nm as shown in Figure 5. Free N-dansyl-2-oxopyrrolidine is characterized by low emission anisotropy value (A = 0.02) in aqueous solution at pH 6.

The fluorescence enhancement that follows the addition of increasing concentrations of N-dansyl-2-oxopyrrolidine to a fixed concentration of enzyme (15μM) was used to determine the stoichiometry of binding. The results of fluorometric titrations conducted at fixed concentrations (15μM) can be fitted to a single binding constant according to equation (4). The best fit of the data in Figure 6 was obtained with Kd = 6μM.

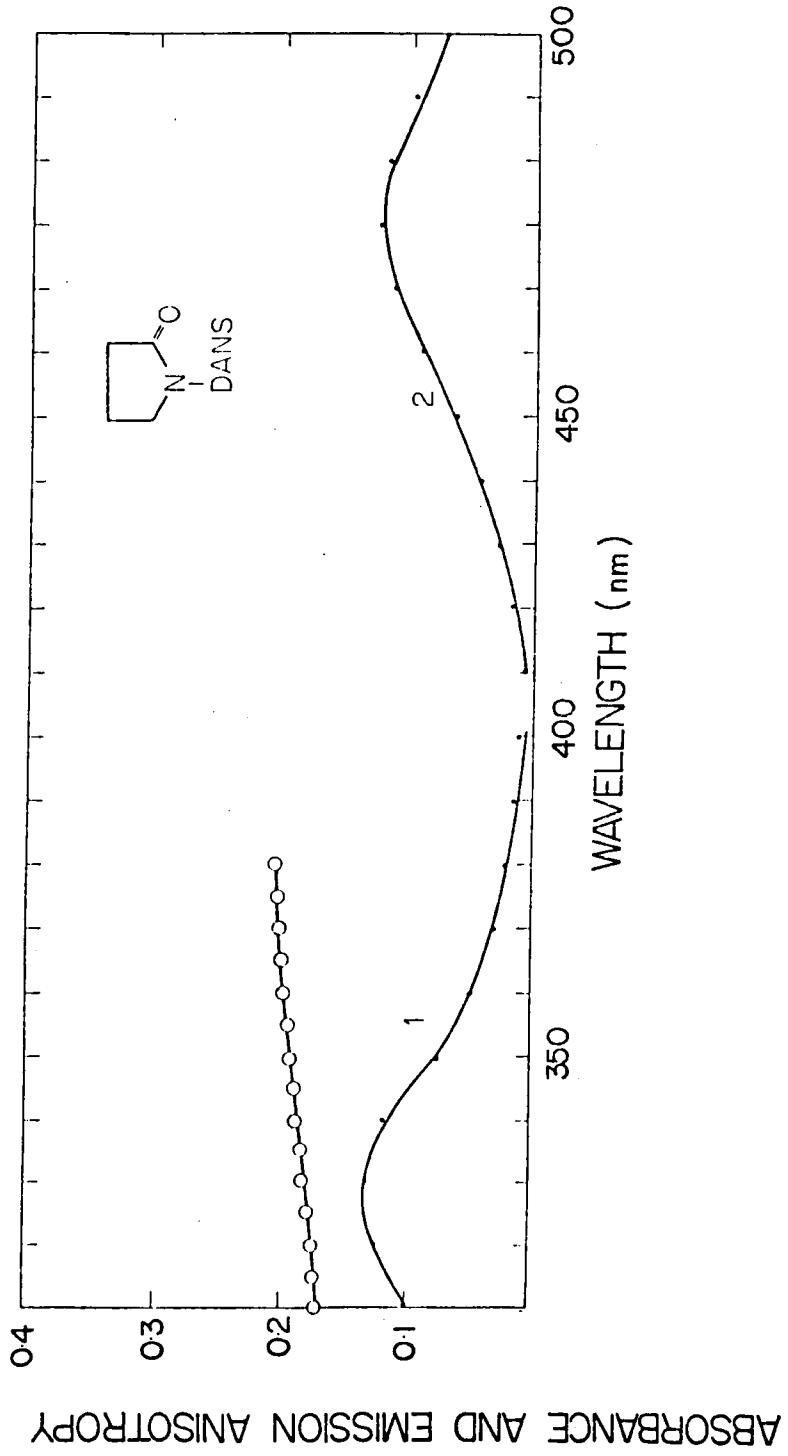

Figure 5. Absorption (1), emission (2) and emission anisotropy (0) spectra of N-dansyl-2-oxopyrrolidine (30μM) in the presence of pyridoxal kinase (50μM) in 70mM potassium phosphate (pH 6) at 25°C.

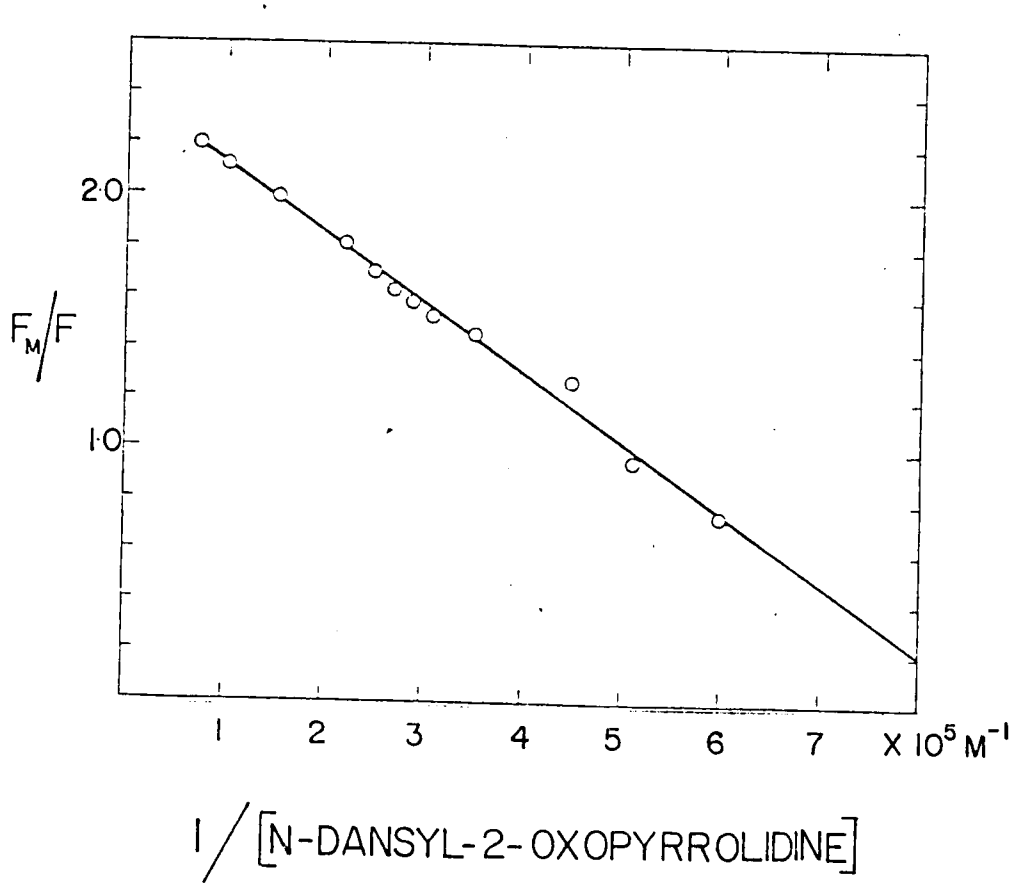

Figure 6. Analysis of the binding results obtained by fluorescence measurements when pyridoxal kinase (15μM) was mixed with increasing concentrations of N-dansyl-2-oxopyrrolidine at 25°C. Plot of F_M/F vs. 1/[N-dansyl-2-oxopyrrolidine] according to equation 3. The dissociation constant is 6μM.

Upon binding to the enzyme, the emission of N-dansyl-2-oxopyrrolidine decays with a fluorescence lifetime of 19 ns. (Figure 7), which can be used to determine the rotational correlation time of the ligand. If the ligand displays any mode of flexibility during the lifetime of the excited state (20 ns.), then the observed rotational correlation time should be shorter than the value expected for a compact macromolecule of the size of pyridoxal kinase. If, on the other hand, the ligand is completely immobilized by the protein, the observed rotational correlation time should be of the same order of magnitude as that of the value expected for the macromolecule.

When the emission anisotropy of N-dansyl-2-oxopyrrolidine in the presence of the kinase was measured as a function of the temperature, it was found that the plot Ao/A against τ/ϕ is linear over the temperature range 8-30°C (Figure 8). Since the rotational correlation time of a spherical protein is related to Ao/A by means of Equation (6)

$$\frac{Ao}{A} = 1 + (\tau/\phi) \qquad (6)$$

where τ is the fluorescence lifetime of the ligand and ϕ is the rotational correlation time. It is possible to determine a rotational correlation time of 35 ns. for bound N-dansyl-2-oxopyrrolidine. A rotational correlation time of this magnitude corresponds to a probe which rotates in common with the protein.

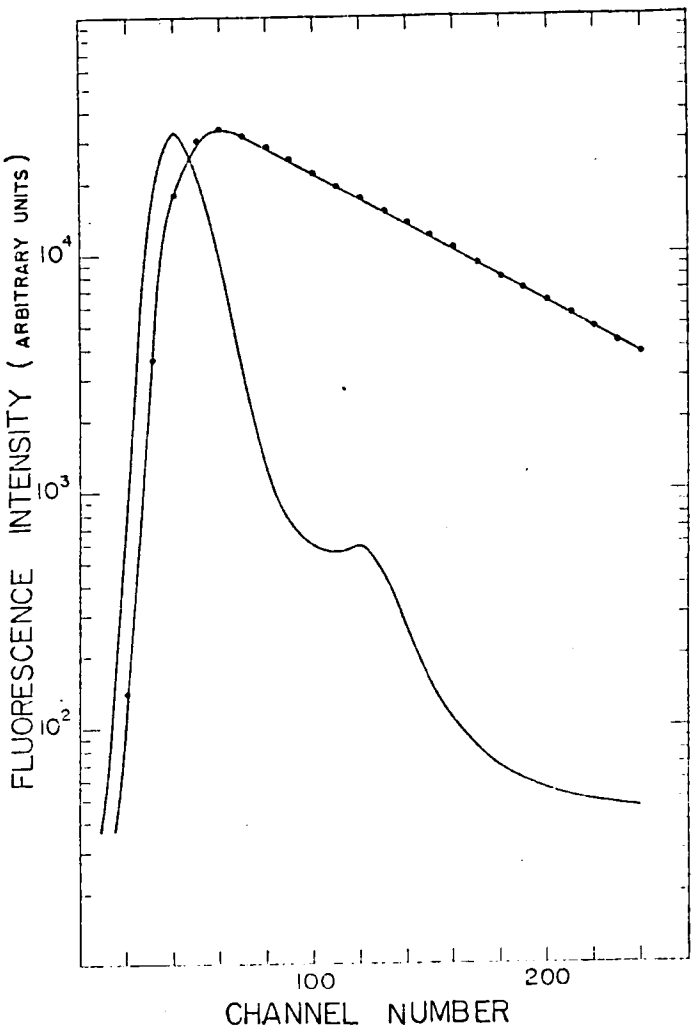

Figure 7. Fluorescence decay curve (●) of N-dansyl-2-oxopyrrolidine (30μM) in the presence of pyridoxal kinase (50μM) in 70mM potassium phosphate (pH 6) at 25°C obtained with the monophotonic fluorescence technique. The lamp profile is included in the figure. A fluorescence lifetime of 19ns was obtained for N-dansyl-2-oxopyrrolidine in the presence of enzyme.

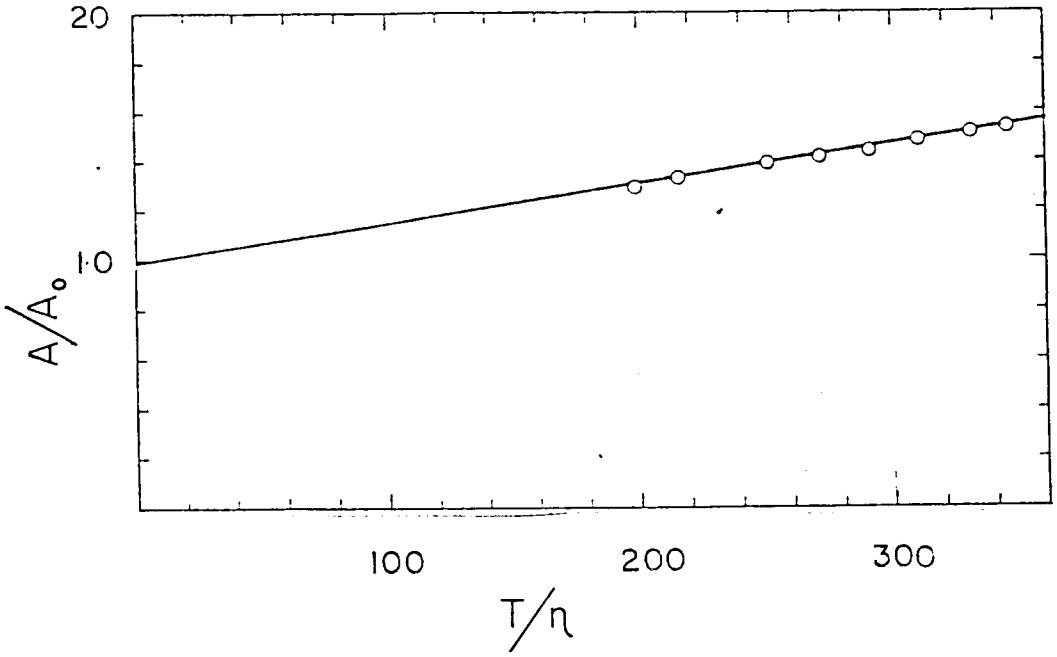

Figure 8. Plot of A_o/A vs. T/η according to Perrin's equation. A rotational correlation time of 35 ns. is obtained for N-dansyl-2-oxopyrrolidine (30μM) in the presence of pyridoxal kinase (50μM) in 70mM potassium phosphate (pH 6). Excitation wavelength 365nm.

C. COMPETITION BETWEEN ATP AND N-DANSYL-2-OXOPYRROLIDINE

The effect of the substrates pyridoxal and ATP on the binding of N-dansyl-2-oxopyrrolidine to pyridoxal kinase was investigated by two independent methods, fluorometric measurements and enzymatic assays.

As illustrated in Figure 9, the addition of pyridoxal (0.3mM) to solutions of pyridoxal kinase (10μM) and N-dansyl-2-oxopyrrolidine (10μM) had no effect on the intensity of the fluorescence observed at 480nm (excitation 360nm). In contrast to pyridoxal, ATP markedly diminished the intensity of the fluorescence emitted by N-dansyl-2-oxopyrrolidine complexed to the enzyme. This is illustrated in Figure 9 where it may be seen that the quenching of N-dansyl-2-oxopyrrolidine is completely suppressed.

Thus, the addition of saturating concentrations of ATP to a solution containing enzyme (10μM) and N-dansyl-2-oxopyrrolidine (10μM) causes dissociation of fluorescent ligand from the non-polar binding site of the protein. Further support for the concept that competition between N-dansyl-2-oxopyrrolidine and ATP takes place was derived from the analysis of initial velocity patterns.

The effect of N-dansyl-2-oxopyrrolidine on the reaction catalyzed by pyridoxal kinase was examined at pH 6 in 70mM potassium phosphate containing 0.1mM $ZnCl_2$ and 0.1mM pyridoxal. With ATP as the variable substrate and N-dansyl-2-oxopyrrolidine as the changing, fixed inhibitor, the initial velocity pattern depicted in Figure 10 was obtained. It is evident that the N-dansyl-2-oxopyrrolidine is a competitive inhibitor with respect to ATP at saturating concentrations of pyridoxal (K_i = 2μM).

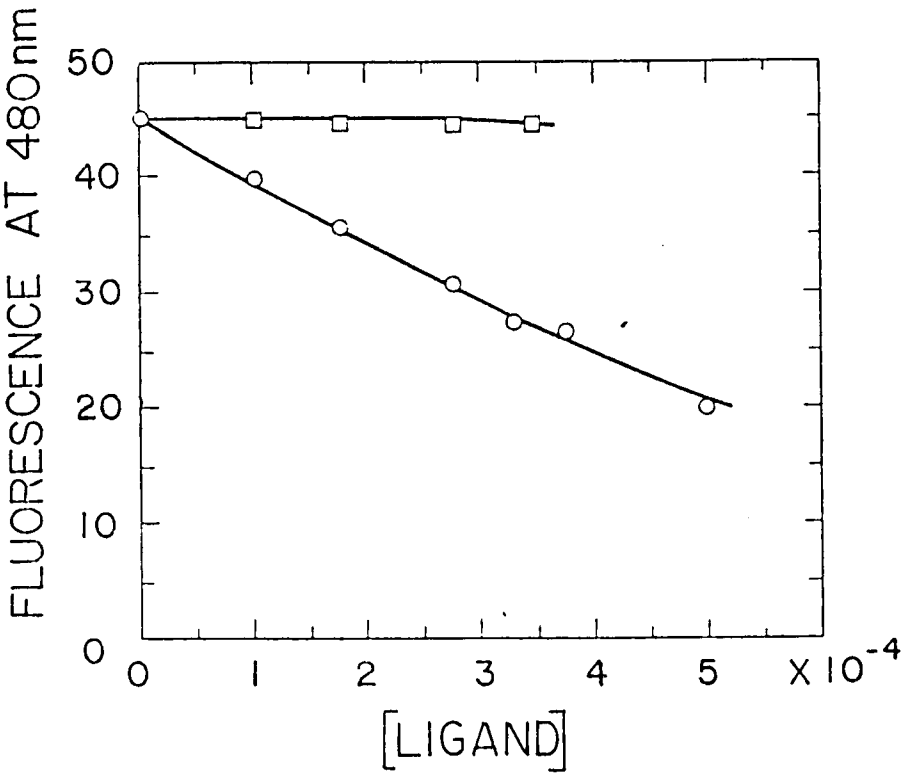

Figure 9. Effect of addition of ATP (O) and pyridoxal (□) on the fluorescence emitted by N-dansyl-2-oxopyrrolidine at 480nm (excitation 365 nm). The concentration of N-dansyl-2-oxopyrrolidine (10μM) and pyridoxal kinase (10μM) were kept constant during addition of either ATP or pyridoxal at 25°C.

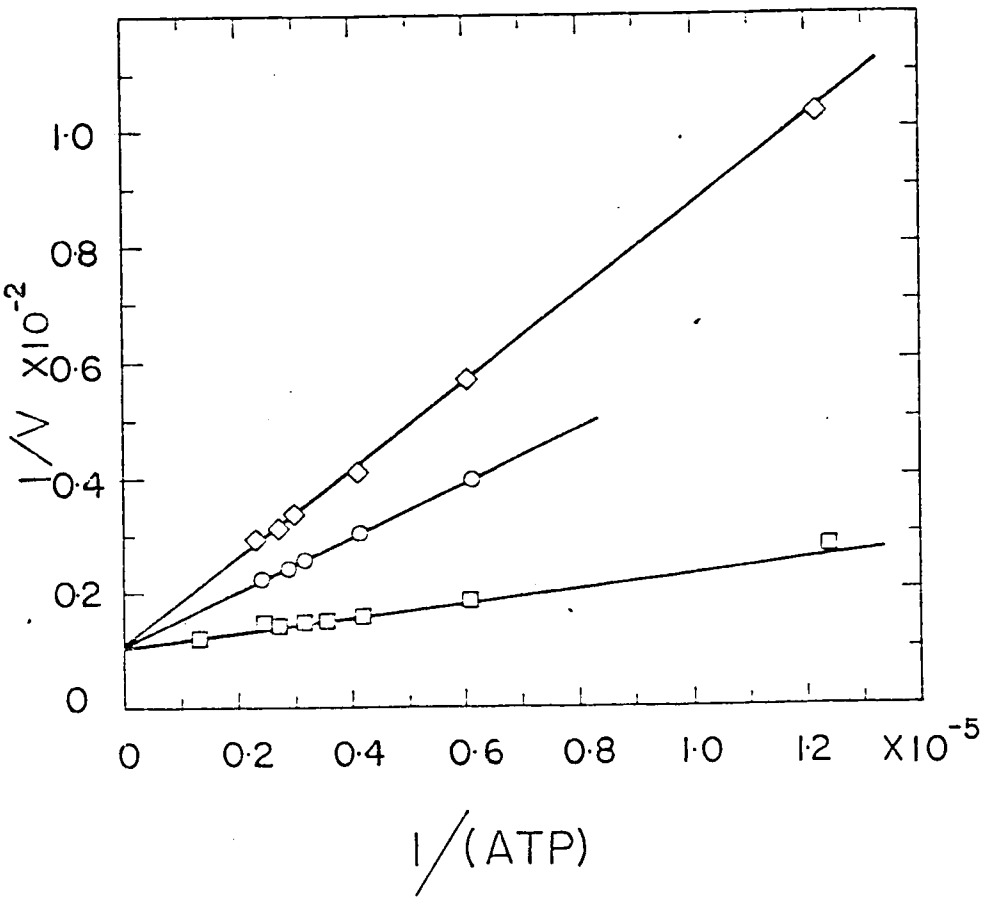

Figure 10. Kinetics of inhibition of pyridoxal kinase by N-dansyl-2-oxopyrrolidine. Lineweaver Burk plots of initial rates of the forward reaction versus [ATP] concentrations in the presence of 0 (□), 1 (○) and 1.5μM (◇) N-dansyl-2-oxopyrrolidine. The concentration of pyridoxal was held constant (0.1mM) in 70mM potassium phosphate (pH 6) containing 0.1mM $ZnCl_2$. Velocity is expressed as change in optical density at 388nm per minute. At the pH optimum of 6, the K_M for pyridoxal is 3×10^{-5}M and the K_M for ATP 2×10^{-5}M.

Discussion

The principal findings herein are firstly, that pyridoxal, a substrate of pyridoxal kinase, binds to the catalytic site of the enzyme with a dissociation constant of 11μM; secondly, N-dansyl-2-oxopyrrolidine, a strong competitive inhibitor with respect to ATP, binds to the nucleotide site of the kinase. A photoselection technique, fluorescence polarization spectroscopy, was used to determine the emission anisotropy values of the substrates trapped by the protein matrix. The emission anisotropy values of bound pyridoxal change from 0.10 to 0.16 as the excitation wavelength coincides with either free or bound pyridoxal. Under similar conditions, the emission anisotropy values of bound pyridoxal are considerably lower than those expected for a fluorophore rigidly trapped by the protein matrix.

The nature of the process leading to a decrease of the emission anisotropy values of bound pyridoxal may be discussed in reference to three mechanisms, which are known to influence the emission anisotropy spectra. Firstly, the enzyme may not be saturated by the substrate; therefore, according to the emission anisotropy law $\overline{A} = \sum_{i}^{n} AiIi$, the presence of free pyridoxal in the system is responsible for a decrease in the observed emission anisotropy ($\overline{A}$). Secondly, the fluorescence lifetime of pyridoxal may be changed by interactions with the enzyme; and thirdly, the rotational mobility of pyridoxal may not be entirely suppressed by the protein matrix.

For a protein containing one binding site, the fraction of ligand bound (α) is related to the fractional saturation of the protein ($\overline{v}$) by the following equation (7)

$$\alpha = \frac{\bar{v}}{\frac{Kd}{[Po]}\frac{\bar{v}}{1-\bar{v}} + \bar{v}} \qquad (7)$$

where Kd is the dissociation constant and [Po] is the total protein concentration. If [Po] > Kd, the first term in the denominator becomes small and the fraction of ligand bound approaches 1, i.e. all the ligand in solution is stoichiometrically bound.

The maximum emission anisotropy value recorded in our experiments (A = 0.16) was obtained at a protein concentration (200μM) which is ten fold greater than the concentration of pyridoxal (20μM) and almost 20 fold greater than Kd (11μM). Hence, the condition [Po] > Kd is satisfied and it seems reasonable to assume that all the molecules of pyridoxal are stoichiometrically bound to the enzyme. In evaluating the effect of the binding on the fluorescence decay time of pyridoxal, it is important to note that the fluorescence quantum yield of pyridoxal remains practically invariant upon addition of enzyme. Furthermore, fluorescence lifetime measurements revealed that free and bound pyridoxal are characterized by the same fluorescence decay time value (τ = 1.5 ns.) when examined by the single photon counting technique.

From these conditions, it follows that the value of emission anisotropy (A = 0.16) corresponding to bound pyridoxal can be attributed to some type of local freedom of motion displayed by the substrate when it is interacting with amino acid residues of the catalytic site.

One can estimate the degree of rotational motion by resorting to Perrin's equation, which allows a determination of the rotational

correlation time expected for an emission anisotropy value of 0.16 at 25°C. For A_0 = 0.32, A = 0.16 and τ = 1.5ns, the rotational correlation time is equal to the fluorescence lifetime.

This value is significantly shorter than the rotational correlation time calculated for a compact macromolecule of 60,000 molecular weight (ϕ = 30ns). It is interesting to note that ADP, an analog and competitive inhibitor of ATP, has little effect on the emission anisotropy spectrum of bound pyridoxal. This observation is interpreted to mean that the rotational mobility displayed by pyridoxal at the level of the catalytic site is not influenced by the binding of compounds to the nucleotide site of pyridoxal kinase.

In marked contrast to pyridoxal, the fluorescence inhibitor N-dansyl-2-oxopyrrolidine is immobilized by strong interactions with an hydrophobic catalytic site of the protein since the apparent rotational correlation time determined by steady emission anisotropy measurement is of the same order of magnitude as the value expected for a macromolecule of 60,000 molecular weight.

N-dansyl-2-oxopyrrolidine is a competitive inhibitor with respect to ATP and the finding that that the fluorescence enhancement produced by the binding of fluorophore can be completely abolished by ATP, indicates that N-dansyl-2-oxopyrrolidine binds at the nucleotide binding site of pyridoxal kinase.

CHAPTER III

PHOTOINACTIVATION OF PYRIDOXAL KINASE

A. PHOTOINACTIVATION

Introduction

Photooxidation was the method chosen to investigate the essential amino acid residues at the active site of pyridoxal kinase. One of the mechanisms of photooxidation is that the photosensitizer, after being energized by illumination with high intensity light, will transfer the absorbed energy to nearby amino acids. Subsequently, the excited amino acids will be oxidized by oxygen molecules available within the enzyme environment. Another mechanism proposed for photooxidation is that the illuminated photosensitizer will transfer its absorbed energy to oxigen molecules in the triplet groupd state and generate oxygen molecules in the singlet excited state. Then, these excited oxygen molecules which have a lifetime of approximately 1μsec, are able to oxidize the amino acids they encounter.

The photodestruction of amino acids required for catalysis will lead to irreversible loss of enzymatic activity of an enzyme and the modified amino acids can be examined by amino acid analysis. Riboflavin, which was not an inhibitor of pyridoxal kinase, was used as the photosensitizer for this experiment.

Materials

Riboflavin, FMN and N-dansyl-2-oxopyrrolidine were purchased from Sigma Chemical Company. Potassium phosphates (monobasic and dibasic) were purchased from Fisher.

Method

Photoinactivation experiments were carried out in a temperature-controlled cell-compartment. The sample of enzyme (2ml) in a glass curvette (1cm path length) was illuminated from the side with a 150 watt mercury lamp. The wavelength of excitation was selected by a Corning glass filter (C-S-0-52) which transmits light at wavelengths longer than 340nm. The energy of exciting light was measured by a thermopile (Model 65, radiometer thermopile, Yellow Spring Instrument Company).

Enzymatic assays were performed as previously described on page 3.

The amino acid composition of native and photoinactivated pyridoxal kinase was determined by Dr. John Massey in Baylor University, College of Medicine, Houston, Texas and Dr. David Lin at the University of Tennessee, Memorial Research Hospital, Knoxville, Tennessee.

Acid hydrolysis of the protein was carried out in HCl at 110°C in a sealed and evacuated tube for 24 hours. The amino acid leucine was used as reference in calculating the number of photooxidized residues in the samples of the inactivated enzyme.

Results

For the photooxidation experiments, samples of enzymes (10μM) in 70mM potassium phosphate buffer (pH 6) were mixed with one, two and five molar excess of riboflavin and irradiated with light of wavelengths longer than 345nm at 10°C. Under this set of experimental conditions, the enzyme is soluble, no turbidity was observed during photoirradiation and the samples were suitable for spectroscopic studies.

Figure 11 shows the results obtained upon exposure to light of a mixture containing enzyme (10μM) and riboflavin (10μM), where it may be seen that in the presence of equimolar concentrations of riboflavin, the enzyme undergoes a loss of enzymatic activity which follows first order kinetics. The addition of the substrate ATP (1mM) to the mixture containing enzyme and riboflavin has a small effect on the rate of inactivation as shown by the results included in Figure 11.

During the photoinactivation experiments, a small decrease in the absorbance of the sensitizer (riboflavin) was detected, and the decrease in absorbance at 455nm as a function of the time of irradiation follows the pattern included in Figure 11. Similar changes in absorbancy at 360nm and 455nm were recorded during irradiation of riboflavin in the absence of enzyme, indicating that the absorbance changes at either 360 or 455nm are entirely due to photodestruction of the sensitizer.

Experiments conducted in several laboratories have shown that histidine, and to a certain extent tyrosine and tryptophan, are the amino acids most susceptible to chemical modification upon illumination in the presence of flavins (32).

Since the loss of enzymatic activity of the photooxidized enzyme might be brought about through chemical modifications of histidine and aromatic amino acid residues, it was thought desirable to determine the amino acid composition of samples of native and photo-irradiated pyridoxal kinase. The results of the amino acid analysis showed a progressive decrease in the histidine content as the irradiation of the enzyme was allowed to proceed for 30 minutes (Table IV). The number of cysteine, tyrosine and tryptophan residues were investigated extensively.

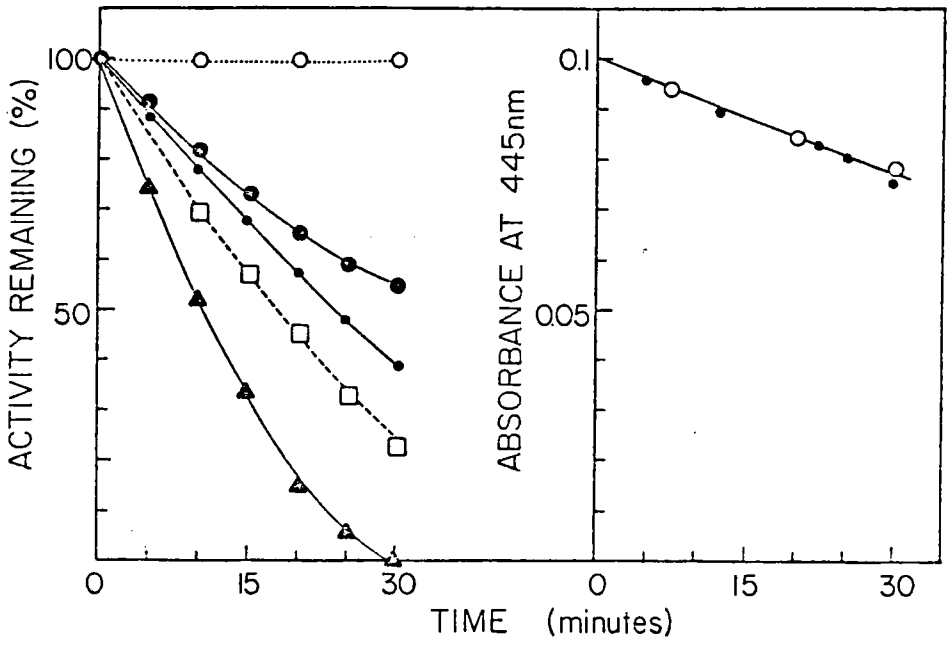

Figure 11. Kinetics of inhibition of pyridoxal kinase by photoirradiation. Left. Loss of pyridoxal kinase activity during irradiation. Enzyme (10μM) irradiated in the presence of riboflavin (10μM) (▲), enzyme (10μM), irradiated in the presence of riboflavin (10μM) and ATP (1mM) (◪), enzyme (10μM) irradiated in the presence of riboflavin (10μM) and DANS-4-aminobutyrate (50μM) (●) and enzyme (10μM) irradiated in the presence of riboflavin (10μM) and β-carotene (20μM) (◐) at pH 6 in 70mM potassium phosphate with an intensity of light of 9×10^5 ERGS Cm^{-2} sec^{-1}. Pyridoxal at a concentration of 0.2mM does not protect photoinactivation of pyridoxal kinase. The enzymatic activity of a solution of enzyme (10μM) and riboflavin (10μM) kept in the dark (○) is included in the figure. Experiments conducted at 10°C. Right. Change in absorbance at 445nm of a solution of riboflavin (10μM) in the absence (◐) and presence (○) of pyridoxal kinase (10μM) (○) upon irradiation with light of 9×10^5 ERGS cm^{-2} sec^{-1}.

TABLE IV

PHOTOINACTIVATION OF PYRIDOXAL KINASE IN THE PRESENCE OF RIBOFLAVIN

Irradiation Time (Minutes)	Activity (%)	Residues per 60.000 Molecular Weight				
		Sulfhydryl	Tryptophan[1]	Tyrosine	Phenylalanine	Histidine
0	100	11.0	6	16	23	11.0
30	0	11.2	5.6	15.8	23.2	9.6

[1] Determined by the colorimeter method of Spies and Chambers (13) and by reaction with N-bromosuccinimide (14).

Cysteine residues were titrated by dithiobisnitrobenzoic acid (DTNB) in the presence and absence of 6M guanidine HCl. Eleven cysteine residues were titrated in the denatured enzyme. However, only one cysteine residue was titrated by excess DTNB without any loss of pyridoxal kinase activity. The kinetics of the reaction of the thiol groups of the enzyme with DTNB was followed. It is evident that the profile of the reactivity curve is biphasic, exhibiting an initial rapid increase in absorbance which is followed by a slower increase in absorbance at 412nm. However, the reactivity of the two classes of thiol groups has no effect on the catalytic activity of the enzyme as demonstrated by the results included in Figure 12.

The reaction of tetranitromethane with the tyrosine residues of pyridoxal kinase was studied under experimental conditions that decrease the radioactivity of thiol groups (33).

The nitration of pyridoxal kinase with 30 fold molar excess of tetranitromethane at pH 8 was followed by measuring the increase in absorbance at 428nm as a function of time. Under these conditions, the reaction is complete within 30 minutes, and the increase in absorbance at 428nm corresponds to the reaction of approximately two moles of tyrosine per mole of enzyme. Despite the nitration of 2 tyrosine residues, the enzyme retains full catalytic activity.

Tryptophan residues were investigated by checking the fluorescence intensity of the tryptophan residues by exciting the pyridoxal kinase at 290nm. Predominantly, there was no change in the intensity of fluorescence from the photo-irradiated kinase from the non-radiated enzyme as shown in Figure 13.

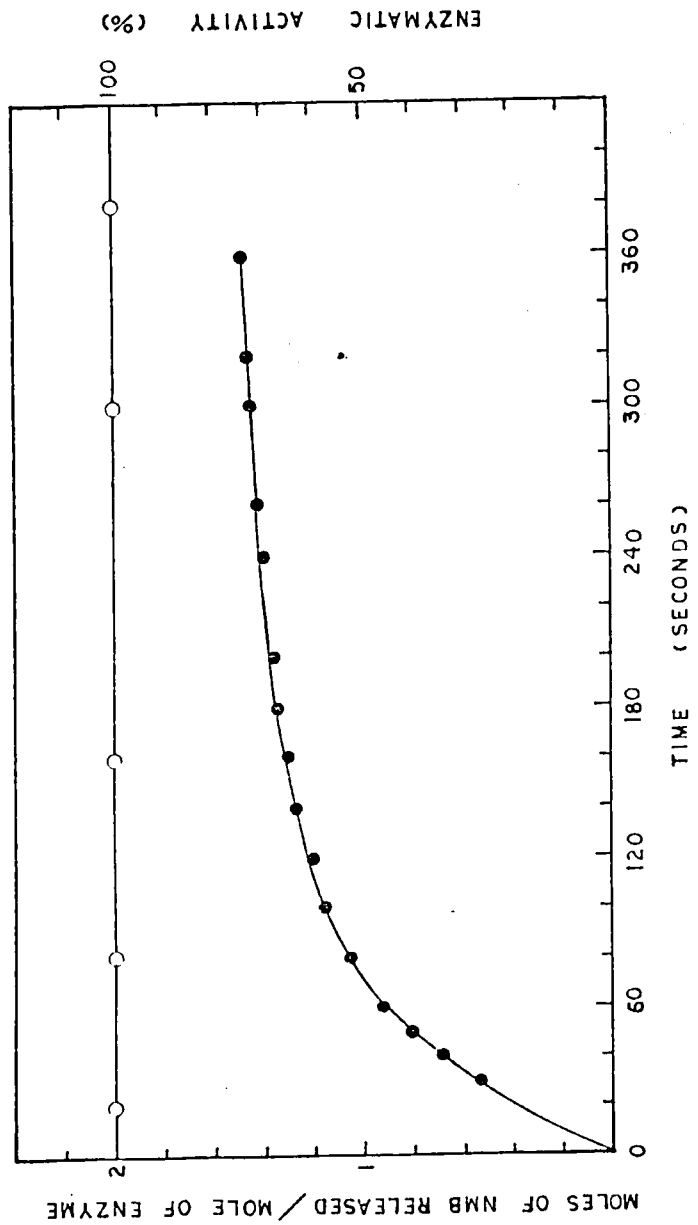

Figure 12. Time course of reaction of pyridoxal kinase (16μM) in 75mM potassium phosphate (pH 7) with 20-fold molar excess of DTNB at 25°C (●). The reaction was followed by measuring the increase in absorbance at 412 nm. Aliquots withdrawn from the incubation mixture were tested for pyridoxal kinase activity (○).

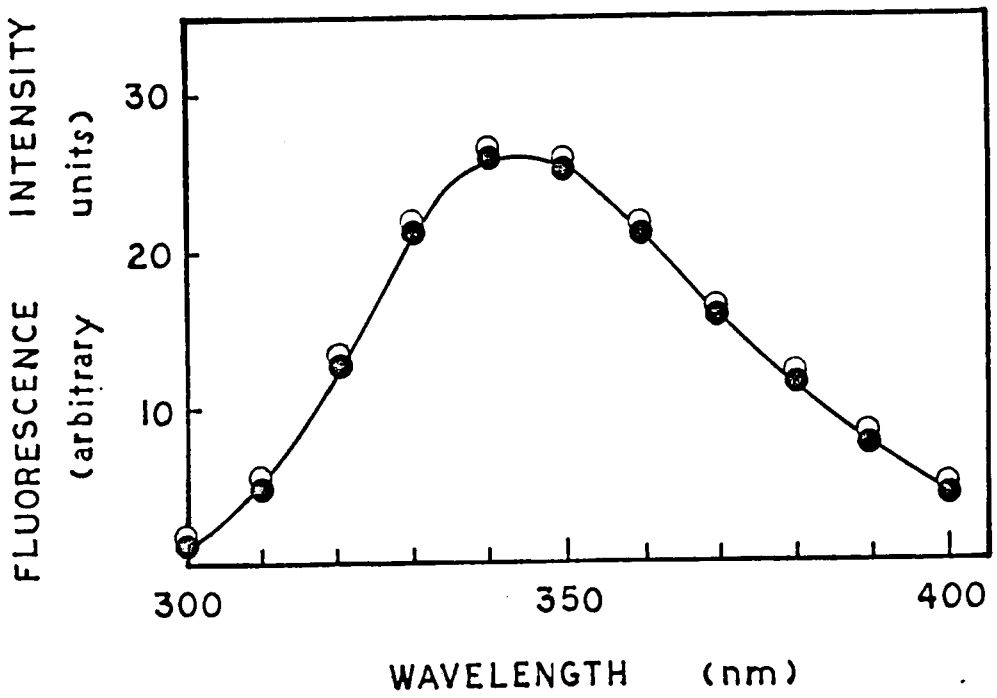

Figure 13. Emission spectra of native (●) and photoinactivated (○) pyridoxal kinase in 70mM potassium phosphate (pH 6). Excitation wavelength 290nm. Both samples have the same absorbancy (A = 0.1) at the exciting wavelength.

B. BINDING OF RIBOFLAVIN

Introduction

Riboflavin is not an inhibitor of pyridoxal kinase. However, binding of riboflavin to the enzyme was observed. The interaction of this ligand's binding to the enzyme outside the catalytic site was studied. The stoichiometry and dissociation constant were determined by emission anisotropy. Subsequently, the mechanism of photooxidation by riboflavin was postulated. Further proof of the presence of singlet oxygen was demonstrated. The experiment was designed to separate the photo-sensitizer and the enzyme during excitation of the sensitizer. Photo-destruction of amion acid residues responsible for enzymatic activity took place without contact between the photo-sensitizer and the enzyme. Therefore, singlet oxygen which has a long lifetime, is very likely to be the candidate for the photo-destruction of the amino acid residues essential for catalytic activity of pyridoxal kinase.

Material

Riboflavin, FMN and N-dansyl-2-oxopyrrolidine were purchased from Sigma. β-carotene was purchased from Calbiochemicals. Phosphatidyl-choline, isolated from egg yolk by column chromatography was a gift from Dr. Leaf Huang.

Methods

Emission anisotropy was conducted as previously described on page 20.

Results

The interaction between pyridoxal kinase and riboflavin was studied by means of fluorescence spectroscopy.

At a protein concentration of 10μM and riboflavin concentration of 10μM, the emission anisotropy of the ligand is increased without any significant change in either fluorescence yield or band position of the emission spectrum. Thus, the emission anisotropy of a mixture containing enzyme (10μM) and riboflavin (2μM) is considerably greater ($\bar{A}$ = 0.23) than the emission anisotropy of free riboflavin (A_F = 0.02) at 25°C.

The change in emission anisotropy associated with the interaction of riboflavin with the kinase was used to determine the stoichiometry of binding. To this end, fluorescence intensity and emission anisotropy ($\bar{A}$) of samples containing fixed concentrations of enzyme and varying concentrations of riboflavin were measured upon excitation with light of 360nm.

As shown in Figure 14, the difference between the observed emission anisotropy ($\bar{A}$) and the emission anisotropy of the free ligand (A_F) increases as the population of free ligand molecules is decreased. This behavior is expected since binding of riboflavin to the protein in solution should restrict the Brownian motion of the ligand with a concomitant increase in the observed emission anisotropy.

Since the fraction of bound ligand (α) is related to ($\bar{A}$), A_F and A_B and β, according to equation (8)

$$\alpha = \frac{\bar{A} - A_F}{(A_B - \bar{A})\beta + (\bar{A} - A_F)} \qquad (8)$$

it is possible to determine α from the titration curve given in Figure 14, by resorting to equation (9)

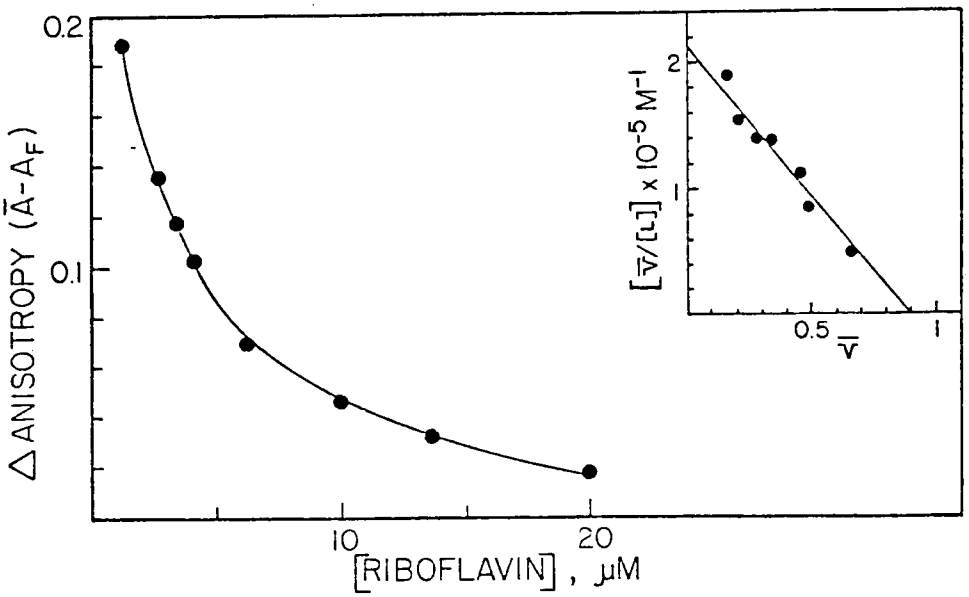

Figure 14. Changes in emission anisotropy ($\bar{A}$ - A_F) upon addition of riboflavin to a fixed concentration of pyridoxal kinase (5μM) in 70mM potassium phosphate (pH 6) at 25°C. Excitation wavelength 360nm.
Insert. Analysis of the binding results. Plot of $\bar{v}/[L]$ vs. $\bar{v}$. $\bar{v}$ is the average number of moles of ligand bound per macromolecule. [L] is the concentration of free ligand (riboflavin).

$$\alpha = \frac{\bar{A} - A_F}{A_B - A_F} \qquad (9)$$

Equation (8) is applicable to the determination of α when the ratio of fluorescence yield for bound (q_B) and free ligand (q_F) is equal to one ($\beta = q_B/q_F$). Since the binding of riboflavin to the enzyme does not elicit any change in the fluorescence yield, the use of the equation is fully justified. When the titration results were analyzed by plotting $\bar{V}/[L]$ vs. $\bar{V}$, it was found that the straight line intersected the abscissa at $\bar{V} = 0.9$ (Figure 14). Thus, there is one binding site on the protein surface to which riboflavin binds with an equilibrium dissociation constant of 5μM.

The binding of riboflavin to the enzyme does not affect the catalytic function of pyridoxal kinase as demonstrated by the fact that Km and Vmax values of the enzyme remain essentially unchanged in the presence of different concentrations of riboflavin more than 500 fold greater than the equilibrium dissociation constant. This finding is interpreted to mean that riboflavin does not interfere with the binding of the substrates pyridoxal and ATP to the catalytic site of the enzyme.

C. MECHANISM OF PHOTOINACTIVATION OF PYRIDOXAL KINASE

Introduction

Since the binding of riboflavin to the enzyme has been observed and the binding site of riboflavin was not located at the catalytic site of the enzyme, it is interesting to investigate the mechanism of riboflavin as a photosensitizer. Several lines of experimental evidence indicated

that singlet oxygen, generated by transfer of excitation energy from the sensitizer to oxygen, was responsible for the photooxidation of histidine residues in proteins. It is highly possible that singlet oxygen is also involved in the photoinactivation of pyridoxal kinase. In order to substantiate this hypothesis, we decided to carry out two sets of experiments. The first experiment was designed to study the effect of efficient quenchers of singlet oxygen on the rate of inactivation of the enzyme. Singlet oxygen, which is characterized by a lifetime of 1μsecond in water solutions, is deactivated by strong quenchers such as β-carotene (32). The second experiment was to demonstrate that even when riboflavin is not bound to the enzyme, the photoinactivation of pyridoxal kinase proceeds through the intermediacy of singlet oxygen.

Materials

Riboflavin, FMN and N-dansyl-2-oxopyrrolidine were purchased from Sigma Chemical Company. β-carotene was from Calbiochemicals. Phosphatidylcholine purified from egg yolk was a gift from Dr. Leaf Huang.

Methods

Phosphatidylcholine vesicles with FMN trapped were prepared according to the method of Huang (33). Liophylized phosphatidylcholine (100mg) was suspended in 2ml of 0.07M potassium chloride containing 1mM FMN. The suspension was ultrasonically irradiated (20 KHZ) under nitrogen at 10°C for 1 hr. and then centrifuged at 105,000g. The resulting supernatant was then subjected to molecular sieve chromatography on a

Sepharose 4B column (15 x 2cm) previously equilibrated with 0.07M potassium chloride at 4°C.

The fractions eluted from the column were monitored at 300 and 445nm by means of absorption spectroscopy. Two major peaks (fraction I and fraction II) were eluted from the column. Fraction I, which is very heterogeneous with respect to both size and shape, was used throughout these studies.

Results

As shown in Figure 11, β-carotene decreased the rate of photoinactivation by riboflavin.

In order to demonstrate that binding of riboflavin to the enzyme was not required for the inactivation of pyridoxal kinase, photoirradiation experiments were conducted in the presence of phosphatidylcholine vesicles carrying the photosensitizer FMN which apparently has identical function as riboflavin.

When samples irradiated for different lengths of time were assayed for enzymatic activity, it was found that the enzyme underwent an irreversible loss of catalytic activity (Figure 15). Samples of pyridoxal kinase mixed with the phosphatidylcholine vesicles remained fully active provided they were not exposed to irradiation as indicated by the results included in Figure 15.

Since the efficient photoinactivation of pyridoxal kinase might be due to FMN molecules released from the vesicles as a result of irradiation damage, we decided to investigate the effect of irradiation on the permeability properties of phosphatidylcholine vesicles.

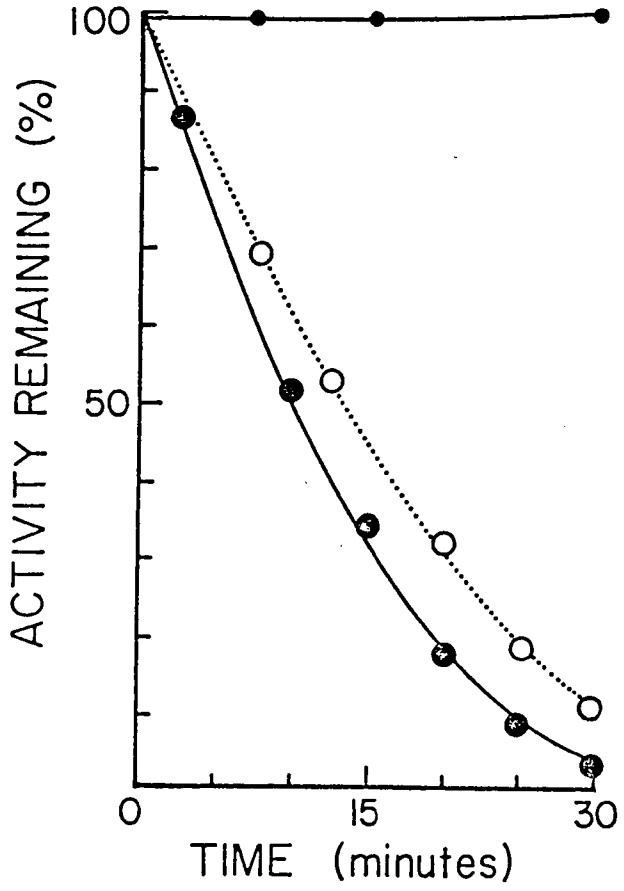

Figure 15. Loss of pyridoxal kinase activity sensitized by FMN. Enzyme (10μM) irradiated in the presence of phosphatidyl choline vesicles containing FMN (30μM) (O) and enzyme (10μM) irradiated in the presence of FMN (30μM) (●) at pH 6 in 70mM potassium phosphate with an intensity of light of 9 x 10^5 ERGS cm^{-2} sec^{-1} sample of pyridoxal kinase (10μM) mixed with phosphatidylcholine vesicles containing FMN (30μM) and kept in the dark (O). Experiments conducted at 10°C.

If disruption of the vesicles has taken place during irradiation, the concentration of FMN inside the vesicles should decrease after exposure to light. If this was the case, chromatography of irradiated vesicles should indicate changes in the elution profile as well as variations in the concentration of FMN. The results of these experiments were included in Figure 16, where it may be seen that rechromatography of irradiated vesicles resulted in a single peak eluting at a volume corresponding to its potision in the original chromatogram.

Furthermore, the absorbance at 455nm due to entrapped FMN was slightly changed after exposure to irradiating light, indicating little or no damage of phosphatidylcholine vesicles under experimental conditions chosen for the photoinactivation experiments.

D. GENERAL DISCUSSION

Using riboflavin as the sensitizer for the photooxidation of pyridoxal kinase, the enzyme is irreversibly inactivated because of the photodestruction of the essential amino acid for catalysis. The results of the amino acid analysis indicate that no change in the cysteine, tyrosine and tryptophan content of the inactivated samples has occurred during the photooxidation process. Thus, it seems reasonable to conclude that the modified histidyl residues play a direct role either in substrate binding or catalysis.

Although there is no direct experimental evidence supporting either alternative, the photoinactivation results suggest that disruption of the ATP binding site has taken place since the rate of photoinactivation

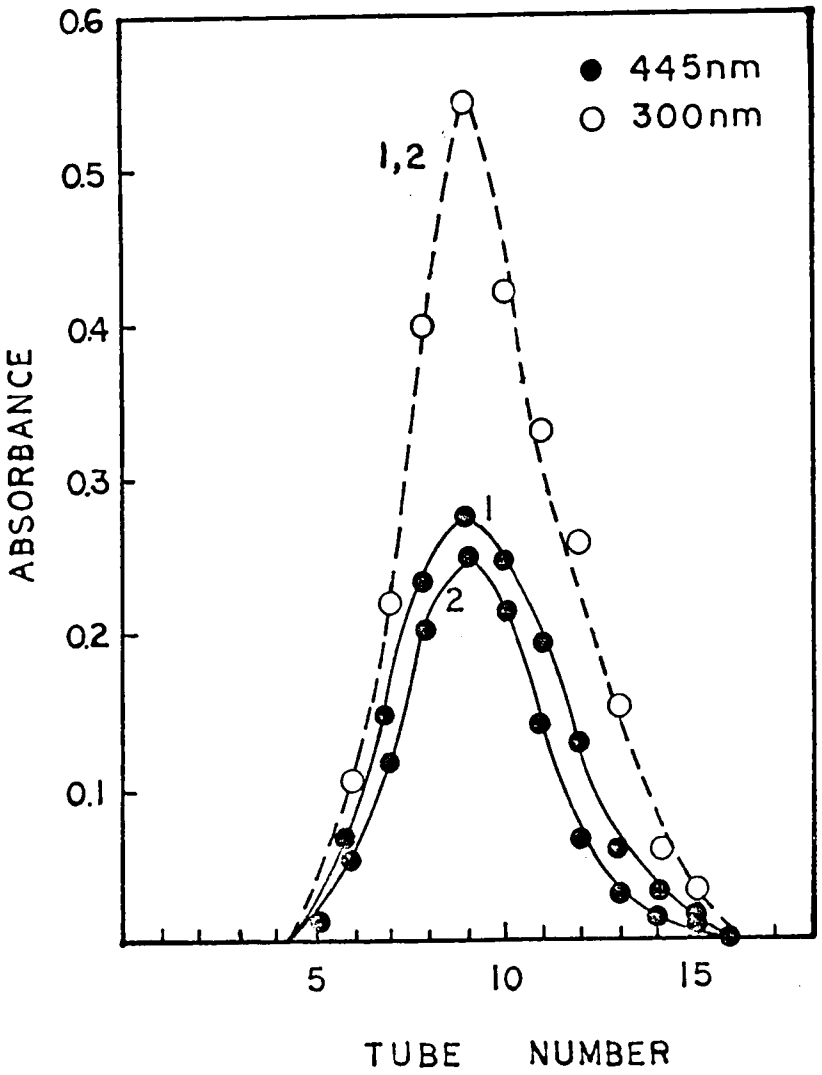

Figure 16. Elution patterns of phosphatidylcholine vesicles containing FMN before (1) and after irradiation (2) for 30 minutes with an intensity of light of 9 x 10^5 ERGS cm^{-2} sec^{-1}. The fractions eluted from a Sepharose-4B column (15cm x 2cm) were monitored at 300 (0) and 445nm (0).

of pyridoxal kinase is influenced by the presence of the substrate ATP or by addition of the strong competitive inhibitor, N-dansyl-2-oxopyrrolidine. No protection against photoinactivation is afforded by the substrate pyridoxal.

The mechanisms underlying the photoinactivation of enzymes are explained in terms of two major classes of reactions in which the triplet state of the photosensitizer is involved (34).

In the second class of reactions, type II mechanism, the triplet state of the sensitizer interacts with oxygen to generate singlet oxygen, which reacts further with amino acid residues of the macromolecule.

It has been demonstrated that singlet oxygen is generated by irradiation of flavins (35), and experiments designed to elucidate the mechanisms underlying the photoinactivation of aspartate aminotransferase have shown that singlet oxygen is the oxidizing agent of histidine residues implicated in catalysis (36).

Since riboflavin is bound to one site of the enzyme distinct from the catalytic binding site, it was thought of interest to demonstrate that the photodynamic action of riboflavin was closely related to its ability to interact with specific sites on the protein. The experiments designed to test this hypothesis have shown that riboflavin acts as an efficient photosensitizer even when it is not interacting with the protein as demonstrated by the photoinactivation results obtained with entrapped FMN. It appears that long lived species (singlet oxygen) diffusing over long distances after being generated by irradiation of the sensitizer are directly involved in the photoinactivation of

pyridoxal kinase. This interpretation is consistent with the protection afforded quenchers of singlet oxygen such as β-carotene.

Although the results do not exclude the possibility that free radicals might be responsible for the photodestruction of histidyl residues in proteins, they give experimental support to the idea that photosensitization could be due, at least in part, to reactions involving singlet oxygen or superoxide ions.

CHAPTER IV

PYRIDOXINE-PHOSPHATE OXIDASE

A. INTRODUCTION

Pyridoxine-P oxidase catalyzes the reactions of

pyridoxine-P + O_2 → pyridoxal-P + H_2O_2

pyridoxamine-P + O_2 + H_2O → pyridoxal-P + H_2O_2 + NH_3

N-(5'-phospho-4'pyridoxal)amine + O_2 + H_2O → pyridoxal-P + H_2O_2 + amine

This enzyme was first found by Pogell from rabbit liver. It was then further studied by Wada and Snell (45) and Korytnyk and co-workers (46) in its partially purified form. The location of this enzyme was reported by Guerrieri (47) to be the cytosol of the cell. Then McCormick et al. purified the oxidase to homogeneity from rabbit liver (48). Most of the information about this enzyme was obtained from the rabbit liver preparation, and no preparation from brain has been reported. In our laboratory, we purified this enzyme to homogeneity from pig brain and have suggested that pyridoxine-P oxidase and pyridoxal kinase both play important roles in the metabolism of vitamin B_6 in brain.

The enzyme was purified with FMN tightly bound. Pyridoxine-P, pyridoxamine-P and N-(5'-phospho-4'pyridoxyl)amine were tested for their activity in the brain preparation. This enzyme seemed to be quite similar to the one obtained from rabbit liver.

The enzyme was found to have a molecular weight of 62,000 and to consist of two identical subunits of 31,000 each. The spectra of the enzyme showed the two absorption maxima of 380nm and 448nm. The enzyme can be resolved into its apo-form and reconstituted to its active form with exogeneous FMN. The effect of structural alterations of FMN were studied by McCormick et al. (48,49) in order to assess the coenzyme specificity of this enzyme.

B. PURIFICATION OF PYRIDOXINE-P OXIDASE

Materials

Pyridoxine, pyridoxamine, pyridoxamine-P, glutathione and pyridoxal-P were purchased from Sigma Chemical Company. Pyridoxine-P was prepared by reduction of pyridoxal-P by sodium borohydride and purified by crystallization. Potassium phosphate (monobasic and dibasic) and ammonium sulfate were from Fisher. Sephadex G-100, CM-Sephadex C-50 and AH-Sepharose were purchased from Pharmacia Fine Chemicals. Hydroxyapatite gel was prepared according to the method of Tiselius (25).

Assay Methods

(I) Pyridoxine-P oxidase activity was measured at 37°C in 0.2M Tris (pH 8) using the method developed by Wada and Snell (45). Pyridoxine-P, excess FMN (2 nmole) and 50μg of BSA were added when oxidase preparation with specific activity >90 were being tested. An E_{410} = 23,000 was used for the phenylhydrazine derivative of pyridoxal phosphate.

(II) Pyridoxine-P oxidase activity was measured at 25°C in 0.1M potassium phosphate (pH 8) by monitoring the formation of pyridoxal-P which has an absorption maximum at 388nm. The E_{388nm} = 4900 $cm^{-1}M^{-1}$ at pH 7 was used to calculate the concentration of pyridoxal-P produced. Pyridoxine-P was used as the substrate for enzymatic assay throughout the whole purification process. The enzymatic assay mixture contains 1 x 10^{-4}M pyridoxine-P in 0.1M potassium phosphate (pH 8) in total volume of 1ml. One unit of enzymatic activity catalyzes the formation of 1nmole of PLP per minute.

Purification Method

Pig brains were obtained from East Tennessee Packing Company. The brains were placed in ice as quickly as possible after slaughter and preparation was begun within one hour. Enzyme preparation procedures were carried out generally at 4°C. A Waring Blendor was used to prepare 25° (w/v) homogenate in a solution of 0.075M potassium phosphate (pH 6.8). The homogenate was centrifuged at 10,000g for 30 minutes and the precipitate was discarded. The supernatant was then treated with ammonium sulfate. The precipitate obtained at 40-60% saturation was dissolved in 0.01M potassium phosphate (pH 6.8) containing glutathione (0.1mM) and was then dialysed against the same buffer overnight to remove the salt. The sample was centrifuged after dialysis at 14,000g to remove the debris and was applied to a column (35 x 2.5cm) of CM-Sephadex C-50. Most proteins were not retained by the gel except the red contaminating protein,

the enzymatically active fractions were combined and applied to a column of hydroxyapatite (25 x 2.6cm) previously equilibrated with 0.01M potassium phosphate (pH 6.8). The enzyme was eluted by a linear gradient made with the equilibration buffer (250ml) and the same volume of 0.2M potassium phosphate (pH 6.8) containing 0.1mM glutathione. The enzyme was eluted at a concentration of potassium phosphate of approximately 0.1M.

The fractions containing pyridoxine-5-P oxidase activity were combined and then concentrated by ultrafiltration by using the Amicon diaflo membrane (XM-50) to about 50ml. The sample was then applied to a Sephadex G-100 column (100 x 2.6cm) previously equilibrated with 0.01M potassium phosphate (pH 6.8). This sample was then chromatographed on a column (14 x 0.9cm) of 4-deoxypyridoxal-Sepharose equilibrated with buffer of 0.01M potassium phosphate (pH 6.8). The enzyme is retained by the derivatized Sepharose and eluted by increasing the concentration of potassium chloride in a step-wise fashion. 0.005M, 0.1M and 0.15M KCl were used and pyridoxine-P oxidase activity was eluted at 0.1M potassium chloride (pH 6.8). The last step in the purification is to apply the most active fractions from the 4-deoxypyridoxyl-Sepharose column to a column (10 x 1.5cm) of hydroxyapatite. The elution was done by a step-wise gradient of 0.05M, 0.1M and 0.15M potassium phosphate (pH 6.8). The enzyme was obtained at the 0.1M elution of potassium phosphate buffer (pH 6.8) (Table V).

TABLE V

PURIFICATION OF PYRIDOXINE-5-P OXIDASE FROM PIG BRAIN

The purification of the enzyme was made from 2 Kg of pig brain tissue, wet weight. A unit of activity (U) is defined as that amount of protein which catalyzes the formation of 1 nmol of pyridoxal-5-P per minute at 25°C.

Treatment	Volume (ml)	Protein (mg)	Protein (mg/ml)	Specific Activity U/mg	Total Activity
Homogenate	3,500	49,700	14.2	0.043	2,137
40-60% $(NH_4)_2$ SO_4 Fraction	360	12,960	36	0.14	1,814
CM-Sephadex Fraction	350	11,550	33	0.15	1,733
Hydroxyapatite Fraction	126	1,260	10	0.788	993
Sephadex G-100 Fraction	15	60	4	14.5	870
4-Deoxypyridoxyl-Sepharose	15	10.8	0.72	40	432
Hydroxyapatite Fraction	10	4.6	0.46	75	345

C. CHARACTERIZATION OF THE ENZYME

1. Polyacrylamide Gel Electrophoresis

The purified enzyme was tested for homogeneity by polyacrylamide gel electrophoresis. The enzymatic activity of pyridoxine-P oxidase was detected by activity staining.

Materials

Photo flow 200 was from Eastman Kodak. Acrylamide, N,N', methylene bis-acrylamide, N,N,N',N' tetramethylene diamine, and naphthol blue black was from Eastman Organic. Acetic acid and ammonium persulfate were from Fisher. Nitroblue tetrazolium and phenazine methosulfate were from Sigma.

Methods

Polyacrylamide gel electrophoresis was conducted according to the method described previously (page 9).

Activity on developed gels was visualized by incubating the gels in 0.1M potassium phosphate (pH 8), 1.0mM pyridoxine-P, 0.1mg/ml of nitroblue tetrazolium, 0.05mg/ml of phenazine methosulfate, and 1μM FMN at 37°C for 1 to 3 hours until maroon bands appeared. The activity stain solution was removed by incubation in several changes of ice water.

Results

The polyacrylamide gel showed one protein band stained by Amido Black and the activity band was detected at the same position as the protein band. This means that the enzyme is homogeneous.

Discussion

The enzyme purified from pig brain appears to be homogeneous as determined by polyacrylamide gel electrophoresis.

The molecular weight of pyridoxine oxidase was measured by sucrose density gradient centrifugation as shown in Figure 17. The enzyme was also subjected to tests to verify its spectroscopic properties and substrate specificities.

2. SDS Polyacrylamide Gel Electrophoresis

Materials

Photo flow 200 was from Eastman Kodak. Acrylamide,N,N'methylene bis-acrylamide, N,N,N',N'tetramethylene diamene and naphthol blue black were from Eastman Organic. Sodium dodecyl sulfate, acetic acid and sodium phosphate (monobasic and dibasic) were from Fisher.

Methods

Sodium dodecyl sulfate gel electrophoresis was performed in a slab gel electrophoresis designed in our laboratory. Slab gel was prepared by the method of Osborn and Weber (50). Slab gel of size 825mm x 900mm x 3mm was prepared by 7% polyacrylamide and the gel buffer (0.2M sodium phosphate pH 7.2 with 0.2% of sodium dodecyl sulfate). The gel was polymerized by using ammonium persulfate solution. After the installment of the slab gel into the electrophoresis apparatus, the upper and lower reservoir were filled by buffer containing 50% of the gel buffer in water. Samples, before being applied to the process, had to be dialyzed against 0.01M sodium phosphate pH 7.2

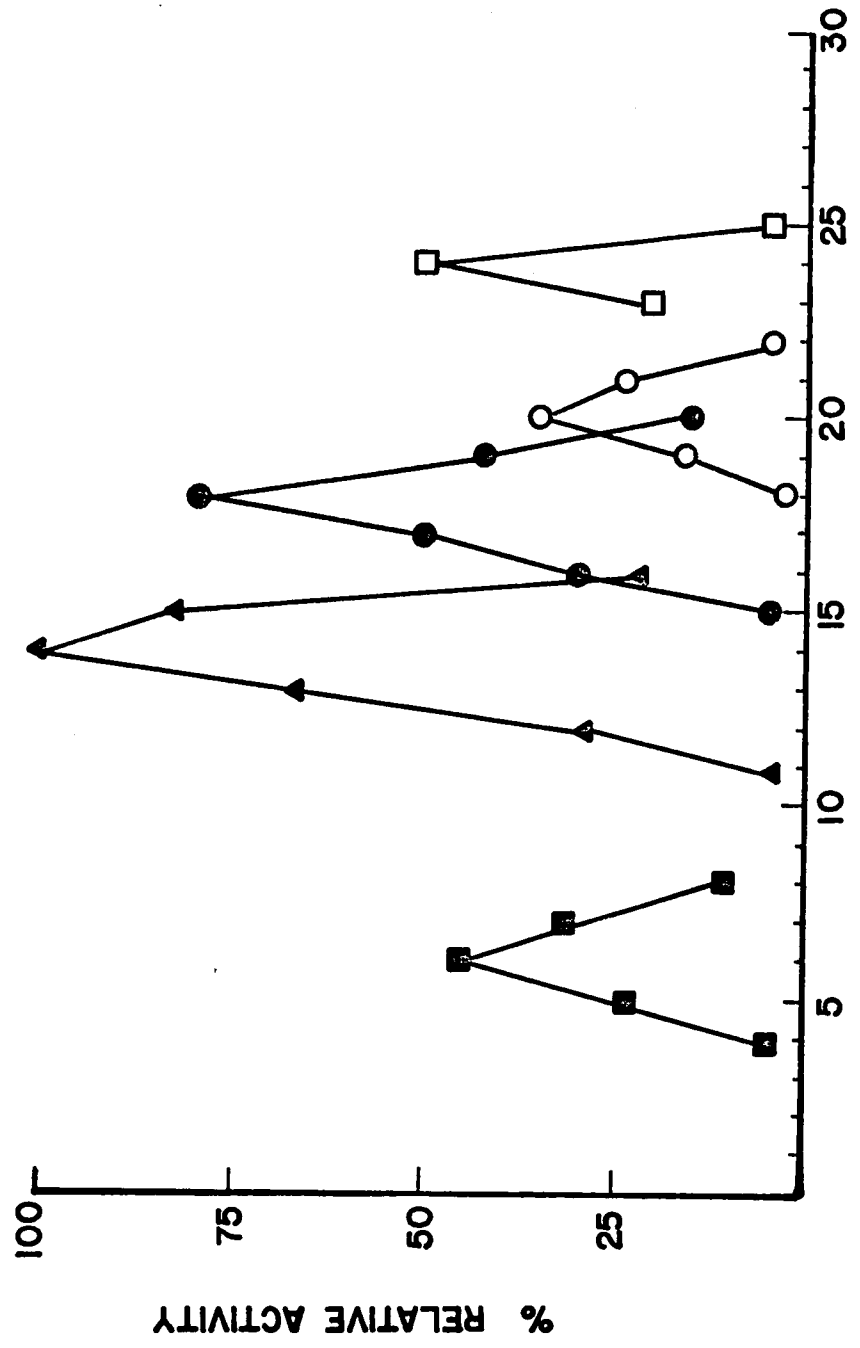

Figure 17. Sucrose density gradient centrifugation of pyridoxine-P oxidase for molecular weight determinations. Standard proteins used in the centrifugation were [■] catalase, MW 240,000, [▲] lactate dehydrogenase, MW 142,000, [●] alkaline phosphatase, MW 80,000 and [□] lysozyme MW 17,200. [○] pyridoxine-P oxidase was calculated to have a molecular weight of 60,000.

in 0.1% SDS and 0.1% 2-mercaptoethanol. The samples, after dialysis, were applied with two drops of bromophenol blue as the tracking dye. Sixty mA was applied and the whole procedure took about 10 hours to complete.

Results

The SDS-polyacrylamide gel electrophoresis showed one band. As compared with other protein standards, pyridoxine-P oxidase has a molecular weight of 31,000. This is consistent with the results as published by McCormick (48) that pyridoxine-P oxidase consists of two identical subunits. Each subunit of pyridoxine-P oxidase is of 31,000 of the molecular weight as shown in Figure 18.

Discussion

The enzyme had its purity and molecular weight determined by SDS-gel electrophoresis.

D. SPECTRA AND SUBSTRATE SPECIFICITY OF THE ENZYME

Materials

Pyridoxine-P was prepared as previously described on page 61. Pyridoxamine-P was purchased from Sigma Chemical Company. Pyridoxyl-amino acids were prepared by reducing a mixture of pyridoxal-P and amino acids by $NaBH_4$ and the phospho-pyridoxyl derivatives were isolated by ion-exchange chromatography.

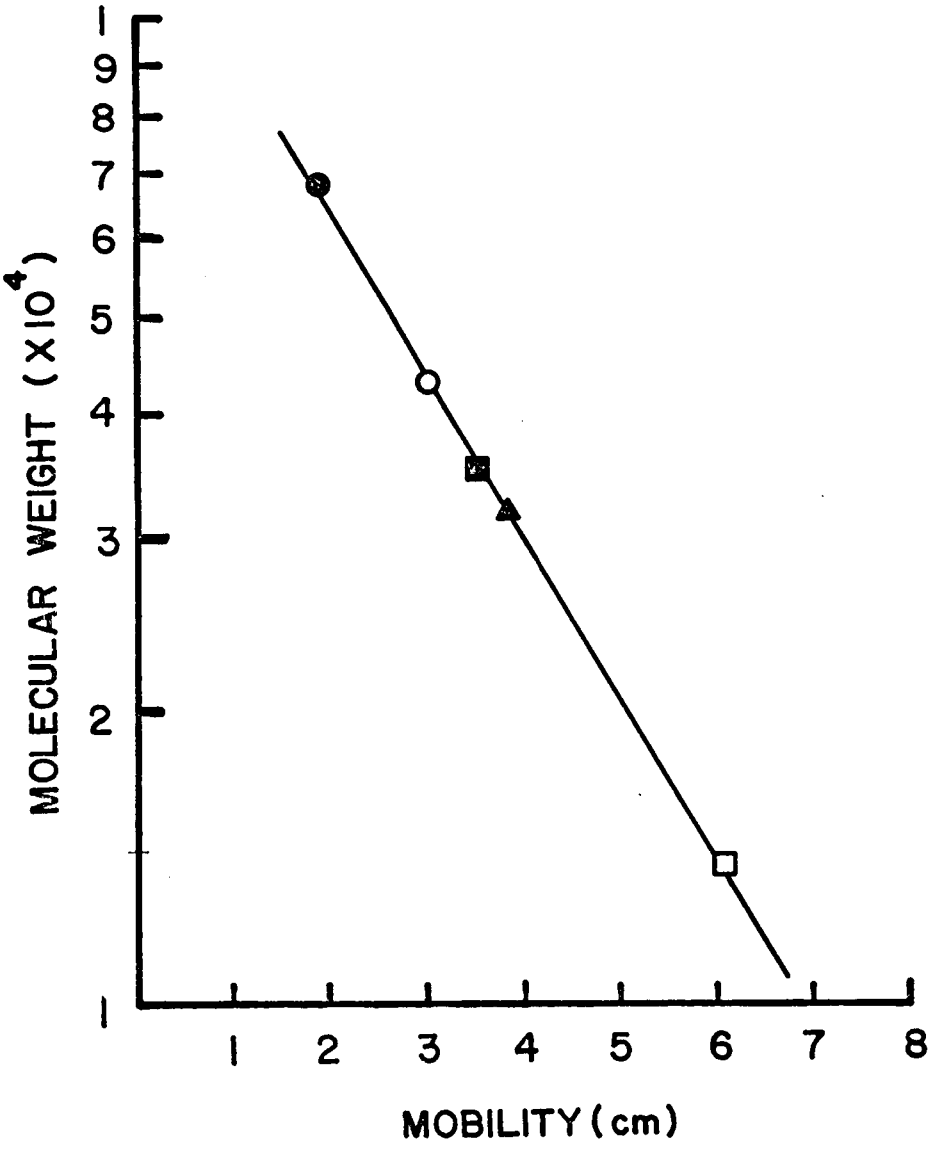

Figure 18. SDS-polyacrylamide gel electrophoresis of pyridoxine-P oxidase [▲]. Standard proteins used for molecular weight determination were [●] BSA MW 68,000, [O] ovalalbumin MW43,000, [■] pepsin MW 34,600 and [□] lysozyme MW 14,300. Pyridoxine-P oxidase was determined to have a molecular weight of 31,000 for each subunit.

Methods

Spectroscopic measurements of pyridoxine-P oxidase were performed in the Aminco DW-2 spectrophotometer. Assay of the enzyme was performed in the method previously described on page 3.

Results

The absorption spectrum of the cofactor FMN, which is firmly bound to the protein, exhibits two absorption bands centered at 380nm and 445nm, respectively (Figure 19). The absorption band at 380nm is shifted towards longer wavelengths when compared with the absorption band at 372nm characteristic of free FMN at pH 7. The ratio of absorbances A_{380}/A_{445} = 1.2 for the holoenzyme is significantly higher than the ratio of A_{372}/A_{445} = 0.9 for free FMN. Reduction of the enzyme by dithionite results in the loss of both absorption bands centered at 380 and 445nm.

Pyridoxine-5-P oxidase displays an emission maximum at 332nm when excited at either 280 or 290nm, indicative of fluorescence emitted by tryptophan residues. The band position of the emission spectra as well as the fluorescence quantum yield of the protein remain essentially invariant when the pH of the solution is increased from 5 to 8. Dissociation of the cofactor FMN brings about a substantial increase in protein fluorescence yield without any change in the band position. The enzymatic activity of the resolved oxidase (apoenzyme) is restored upon addition of increasing concentration of FMN (see page 77). The binding of 1 molecule of FMN per 60,000g of protein brings about more than 80% recovery of catalytic activity as shown by the results included

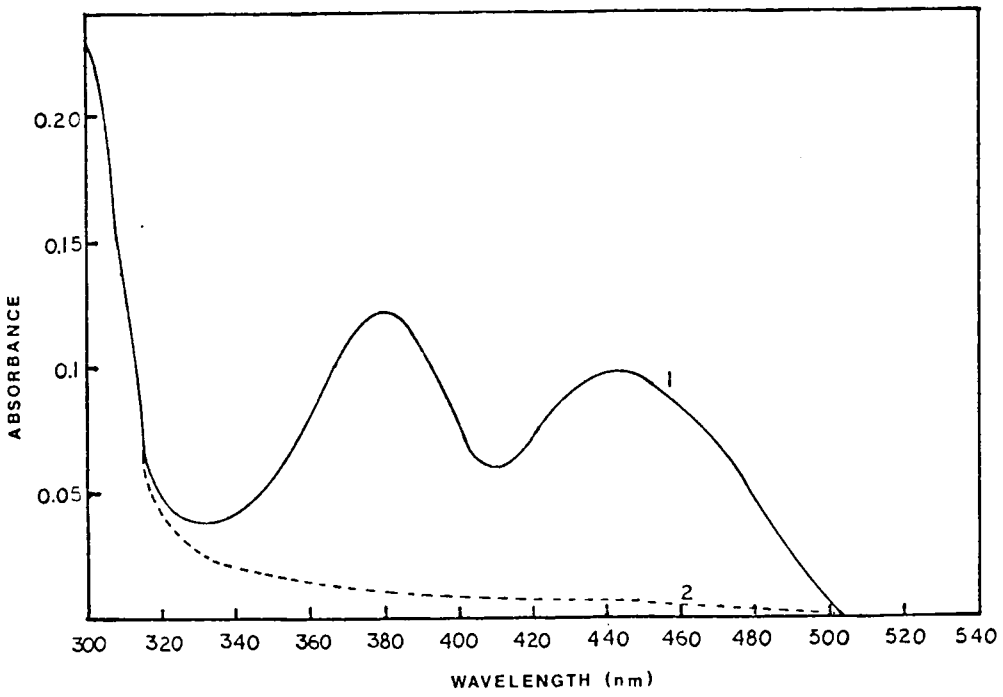

Figure 19. Absorption spectra of pyridoxine-5-P oxidase. Absorption spectrum of the apoenzyme (10μM) (2) and the holoenzyme (10μM) (1) in 0.1M potassium phosphate (pH 7).

on page 77. Although these results indicate that the affinity of FMN for the protein is considerably lower than 10^{-7}M, they do not convey any information about the mechanism of the binding process.

In an attempt to define the specificity of the reaction catalyzed by pyridoxine-P oxidase, the oxidation of the substrates pyridoxine-P, pyridoxamine-P and pyridoxyl-β-alanine were studied at pH 8.4, the optimum pH of the enzyme. The results obtained with all the substrates tested are included in Table V (page 64). The oxidation of pyridoxine-P as catalyzed by the oxidase proceeds with a specific activity of 9 units/mg of protein. Thus, pyridoxamine-P is not the preferred substrate of pyridoxine-P oxidase. The enzyme also catalyzes the oxidation of P-pyridoxylalanine and P-pyridoxyl-β-alanine, but the specific activity is lower than that obtained for pyridoxine-P. P-pyridoxal residues attached covalently to polylysine and reduced aspartate aminotransferase are not oxidized in the presence of the oxidase.

No oxidation of the unphosphorylated derivatives, pyridoxine, pyridoxamine and pyridoxylalanine could be detected over a wide range of pH values (51,52).

E. INHIBITOR OF THE ENZYME

Material

Pyridoxal-P was purchased from Sigma Chemical Company.

Method

Pyridoxine-P oxidase was assayed as previously described on page 3.

Results

It has been shown previously (48) with purified preparations of enzyme from rabbit liver that P-pyridoxal oximes are powerful inhibitors of oxidase activity. This is also true of the purified preparation from pig brain which is inhibited by P-pyridoxaloxime with a K_I of 10μM (Table VI).

The most significant result of the inhibition studies is the finding that pyridoxal-P, a product of the reaction, is a reversible competitive inhibitor of the enzyme. The initial velocity patterns obtained with pyridoxime-P revealed that pyridoxal-P is a competitive inhibitor with respect to pyridoxine-P (Figure 20).

Discussion

Pyridoxine-P oxidase and pyridoxal kinase are the two enzymes responsible for the production of the active form of vitamin B_6, pyridoxal-5-P. Pyridoxal-5-P was observed to inhibit pyridoxine-P oxidase and pyridoxine-P inhibited pyridoxal kinase. Since the conversion of pyridoxine to pyridoxal-5-P requires the activities of the two enzymes, the product inhibitions of the two enzymes may be significant for the regulation of pyridoxal-5-P production in physiological conditions.

F. RESOLUTION OF PYRIDOXINE-P OXIDASE

Materials

Ammonium sulfate, HCl and potassium phosphate (monobasic and dibasic) were purchased from Fisher. Glutathione was from Sigma Chemical Company.

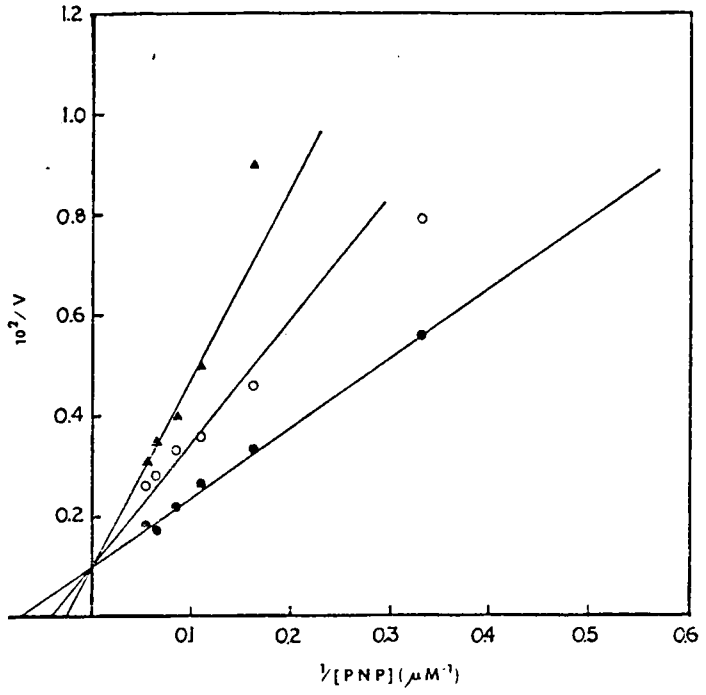

Figure 20. Kinetics of inhibition of pyridoxine-5-P oxidase by pyridoxal-5-P. Lineweaver Burk plots of initial rates of the forward reaction versus pyridoxine-5-P concentrations in the presence of 0 (●), 17 (○) and 42μM (▲) pyridoxal-5-P in 0.1M potassium pyrophosphate (pH 8.4). Velocity is expressed as change in absorbance at 388 nm/minute. The K_I for pyridoxal-5-P is 2.3×10^{-5}M.

TABLE VI

SUBSTRATES AND INHIBITORS OF PYRIDOXINE-5-P OXIDASE

Compound	K_M or K_I (M)	Relative Vmax [1]
Pyridoxine-5-P	1.3×10^{-5}	100
Pyridoxamine-5-P	1.6×10^{-5}	12
P-Pyridoxyl-Alanine	---	6
P-Pyridoxyl-β-Alanine	2.5×10^{-4}	37
P-Pyridoxyl Polylysine	---	0
Aspartate Aminotransferase (Reduced)	---	0
Pyridoxal-5-P	2.3×10^{-5}	

[1] Vmax values are given relative to pyridoxine-5-P as substrate.

Methods

To a solution containing pyridoxine-P oxidase (3mg/ml) in 0.1M potassium phosphate (pH 6.8) was added ammonium sulfate to a final concentration of 0.32g per ml at 4°C. The protein solution was then dialyzed against a solution containing 0.32g per ml of ammonium sulfate adjusted to pH 3.5 by addition of HCl. The dialysis was allowed to proceed at 4°C for 3 hours. The protein solution was then dialyzed against 0.1M potassium phosphate (pH 6.8) for 12 hours at 4°C.

Results

The resulting apoenzyme is inactive and regains 80% of the original enzymatic activity upon addition of FMN as shown in Figure 21.

Discussion

This method of resolution is a modification of the method of Warburg and Christian (53). Another method used by Mayhew (54) modified by Kazarinoff and McCormick (49) by using 2M KBr (pH 4) at 4°C can be used effectively on this brain preparation in order to obtain apoenzyme. However, the method of using ammonium sulfate is more effective and requires less time for the resolution.

DISSOCIATION OF FMN FROM THE HOLOENZYME

Material

FMN was purchased from Sigma Chemical Company. Pyridoxine-P was prepared by the method previously described.

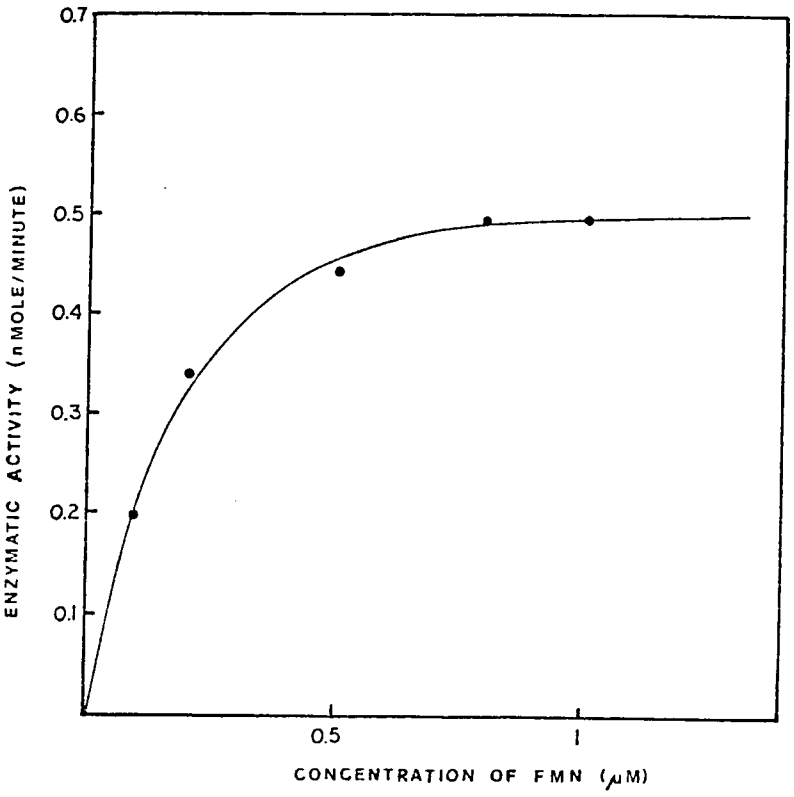

Figure 21. Recovery of pyridoxine-5-P oxidase activity by addition of FMN to the apoprotein of the enzyme pyridoxine-5-P oxidase. Samples of apoenzyme (1μM) were preincubated with increasing concentrations of FMN for 15 minutes at 25°C in 0.1M potassium phosphate (pH 6.8) at 25°C. Aliquots withdrawn from the incubation mixtures were assayed for enzymatic activity at pH 8.0 in the presence of 1mM pyridoxine-5-P.

Method

Pyridoxine-P oxidase was diluted into different concentrations. At low concentration of the enzyme, the cofactor FMN would dissociate from the enzyme. By this method, the percentage of dissociation can be measured by assaying the activity of pyridoxine-P oxidase. A control sample was used by adding excess amount of FMN to the assay mixture in order to compare the percentage of dissociation of the cofactor. The enzyme was assayed at the same condition as previously described (page 3), except incubation of the reaction mixture at 37°C for four hours is required when the enzyme concentration is very low. After incubation, the resulting mixture was tested for enzymatic activity using phenylhydrazine for the quantitation of PLP formed.

Theory

The dissociation constant Kd was determined by equation (10) as

$$Kd = \frac{[EL][L]}{[EL]} \qquad (10)$$

where [E] is the concentration of the apoenzyme, [L] is the concentration of the ligand and [EL] is the concentration of the holoenzyme.

Since $[E_T] = [E] + [EL]$,

$$Kd = \frac{([ET] - [EL])[L]}{[EL]} \qquad (11)$$

$$\frac{Kd}{[L]} = \frac{[E_T] - [EL]}{[EL]}$$

$$= \frac{[E_T]}{[EL]} \quad \frac{[EL]}{[EL]}$$

$$= \frac{[E_T]}{[EL]} - 1 \qquad (12)$$

If $\frac{[EL]}{[E_T]} = f$

then $\frac{Kd}{[L]} = \frac{1}{f} - 1$

Since $[E] = [L]$

therefore $\frac{Kd}{[E_T] = [EL]} = \frac{1}{f} - 1 \qquad (13)$

By substitution,

$$\frac{Kd/[E_T]}{(1 - f)} = \frac{1 - f}{f}$$

$$\frac{Kd}{[E_T]} = \frac{(1 - f)^2}{f} \qquad (14)$$

The graph of $\frac{(1 - f)^2}{f}$ vs. $\frac{1}{[E_T]}$ can be made and Kd can be obtained from the slope of the data points. F value can be determined by assaying the enzymatic activity of the enzyme [EL] after dilution and $[E_T]$ can

be obtained by assaying the diluted enzyme with the addition of an excess amount of FMN to the assay mixture.

Results

The dilution of holo-pyridoxine-P oxidase ranged from 10^{-5}M to 10^{-8}M. The f value was equal to one from 1 x 10^{-6}M to 5 x 10^{-7}M. However, f values lower than one were obtained when the protein concentration varied between 10^{-7}M to 10^{-8}M. The graph of $\frac{(1 - f)^2}{f}$ vs. $\frac{1}{[ET]}$ was plotted and the line extrapolated to the origin as shown in Figure 22. The dissociation constant (Kd) determined from the slope of the line was 9.6 x 10^{-9}M.

Discussion

The dissociation constant of the cofactor, FMN, from the holoenzyme of the brain pyridoxine-P oxidase was 9.6 x 10^{-9}M, which is very close to the value obtained for the rabbit liver enzyme. The FMN, during purification of the enzyme, has been tightly bound to the enzyme and no addition of FMN to enzyme is necessary for the enzymatic assay. In our purification procedure, we obtained holoenzyme because no drastic condition has been applied. However, the purification of the rabbit liver enzyme by Kazarinoff (48) included a step of acetic precipitation which would, in turn, remove the cofactor from the enzyme. Therefore, BSA was required in the assay mixture in order to stabilize the enzyme. The holoenzyme of pyridoxine-5-P oxidase from brain is very stable at 4°C for seven days. Loss in enzymatic activity is detected during the process of freezing and thawing the enzyme preparation.

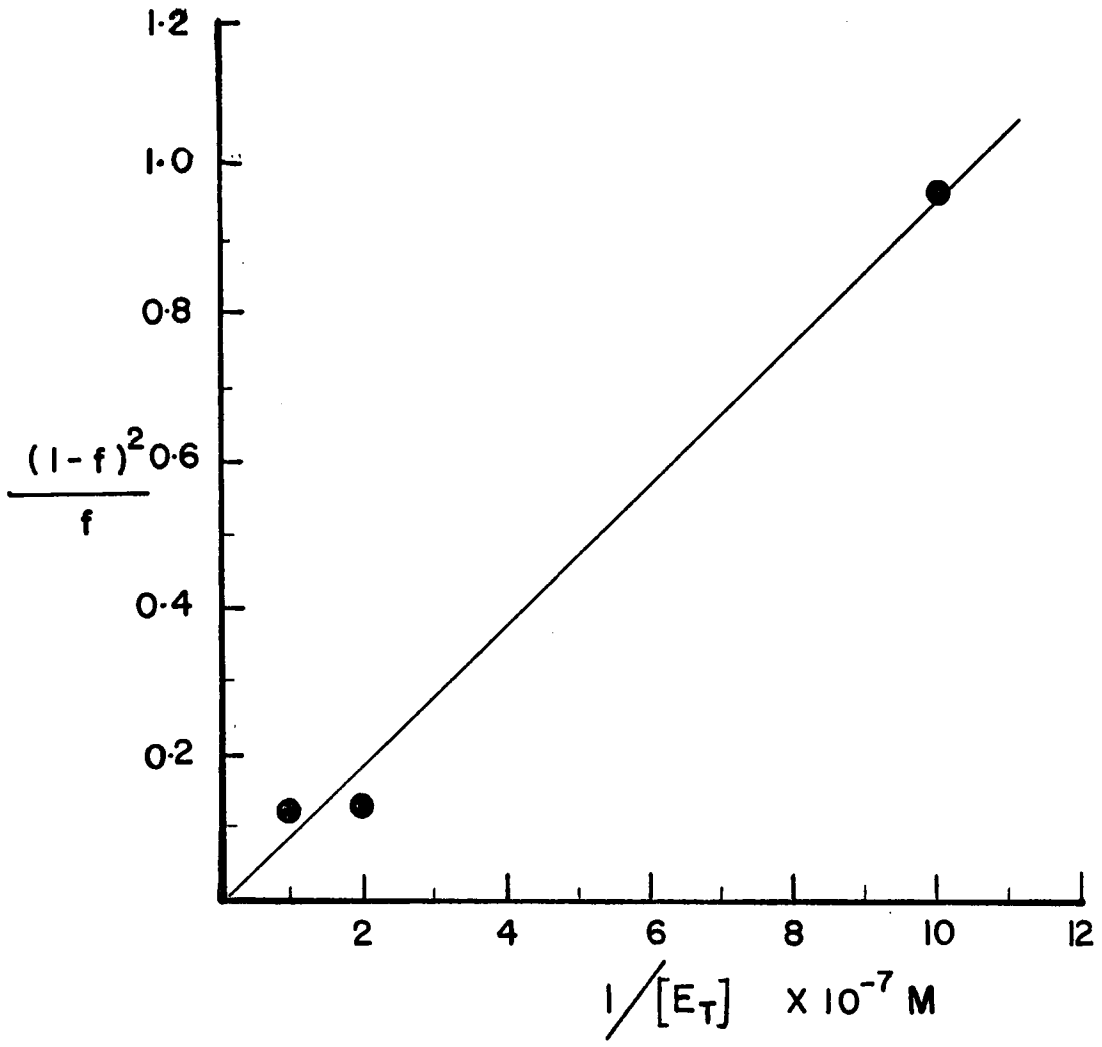

Figure 22. Determination of dissociation constant of FMN from pyridoxine-P oxidase. Kd determined from this graph was 9.6 x 10^{-9}M.

CHAPTER V

INTERACTION BETWEEN PYRIDOXAL KINASE AND PYRIDOXINE-P OXIDASE

A. GENERAL INTRODUCTION

Pyridoxal kinase and pyridoxine-P oxidase catalyze the formation of pyridoxal-P in porcine brain. Due to their similarity in function, it is worthwhile to study the relationship between the two enzymes.

In order to study the protein-protein interaction, fluorescence spectroscopy is the most sensitive method to be applied. The advantage of studying the interactions of the two enzymes by fluorescence spectroscopy is that both enzymes can have their sulfhydryl residues modified by a fluorescent probe without losing any of the enzymatic activities. These sulfhydryl groups can be modified either by 1-5-1 AEDANS or iodoacetamide fluorescein and full enzymatic activities are retained.

Complex formation of pyridoxal kinase and pyridoxine-P oxidase was investigated by resonance energy transfer, polarization of fluorescence and Sephadex G-100 gel filtration. Moreover, interactions between the two enzymes within the complex was also detected by transient kinetic analysis of the coupled assay system.

B. RESONANCE ENERGY TRANSFER

Materials

Iodoacetamide fluorescein was purchased from Molecular Probe (1175, Maple Lane, Loseville, MN 55773). 1-5-1 AEDANS (iodiacetyl-N'-(5 sulfo-1-naphthyl)ethylene diamine was from Aldrich Chemicals.

Methods

Spectral measurements were conducted in a Cary 15 spectrophotometer and Aminco DW-2 double beam spectrophotometer.

Fluorescence measurements were conducted by using the fluorometer designed in our laboratory previously described on page 20.

Polarization of fluorescence was measured in an instrument similar to the one designed by Weber (60).

Theory

When a molecule is energized to the excited state by absorption of energy, it emits energy and returns to its ground state. The emission of energy can be in the form of heat, fluorescence and phosphorescence. If two molecules have the same kind of properties of absorption and emission of energy but they absorb and emit at different wavelengths, a phenomenon which is called fluorescence energy transfer may occur when the absorption maximum of one molecule overlaps the emission maximum of another molecule and also when the two molecules are close together in the range of 20-80Å. The molecule which absorbs is called an acceptor and the other which emits fluorescent light is called a donor. The acceptor will absorb the emission given by the donor to give sensitized fluorescence at a longer wavelength. This dipolar interaction between an electronically excited donor and an acceptor luminophore leading to long-range resonance energy transfer has been described by Forster (37)

$$K_T = \frac{1}{\tau D} \left(\frac{R_0}{R}\right)^6 \qquad (15)$$

where K_T is the rate of energy transfer, τ_D is the excited state lifetime in the absence of the acceptor, R is the interluminophore separation and R_0 is the critical transfer distance of the system. R_0 is dependent on electronic and orientational parameters of the donor-acceptor pair (37).

$$R_0^6 = \frac{9(\ln 10)k^2\phi_D J(\lambda)}{128\pi^5 n^4 N} \qquad (16)$$

Here ϕ_D is the quantum yield of the donor in the absence of the acceptor, $J(\lambda)$ is the overlap integral of the normalized donor fluorescence emission spectrum with the absorption spectrum of the energy acceptor, n is the refractive index of the intervening medium, N is Avogadro's number and k^2 is the orientation factor for dipole-dipole interaction. The overlap integral is given by

$$J(\lambda) = \frac{\int_0^\infty a_m(\lambda)\ F(\lambda)\lambda^4 d}{\int^\infty F(\lambda)d\lambda} \qquad (17)$$

where a_m is the molar absorptivity of the acceptor and $F(\lambda)$ is the relative fluorescent intensity of the donor at wavelength λ. The orientation factor reflects the mutual orientation of donor and acceptor with respect to interluminophore separation vector and is given by

$$k = [\hat{D} \cdot \hat{A} - 3(\hat{D} \cdot \hat{R})(\hat{A} \cdot \hat{R})]^2 \qquad (18)$$

where $\hat{D}$, $\hat{A}$ and $\hat{R}$ are unit vectors along the directions of the transition dipole moment of the donor and acceptor and the separation vector respectively. The limiting values of k^2 are 0 and 4.

Energy transfer results in a decrease in the steady-state fluorescence intensity of the donor in the presence of the acceptor. The ratio of the observed steady-state fluorescence of donor in the absence of (F_D) and presence ($F_{D,A}$) of the acceptor is related to the donor-acceptor separation R and the characteristic distance R_0.

$$\frac{F_D}{F_{D,A}} = 1 + \frac{R_0}{R}^6 \qquad (19)$$

Results

1-5-1 AEDANS (iodoacetyl-N'-(5 sulfo-1-naphthyl)ethylene diamine has an absorption maximum at the wavelength of 360nm and an emission maximum at the wavelength of 450nm. Iodoacetamide fluorescein has an absorption maximum at the wavelength of 485nm and an emission maximum at 520nm. The emission spectrum of 1-5-1 AEDANS overlaps the absorption spectrum of iodoacetamide fluorescein as it was shown in Figures 23 and 24. If energy transfer was allowed from 1-5-1 AEDANS to iodoacetamide fluorescein, sensitized fluorescence at 520nm would be detected by exciting the 1-5-1 AEDANS at 360nm. The critical distance is then determined by the equation proposed by Forster (57) for radiationless energy transfer between singlet excited states.

Using equation 17, the overlap integral was determined to be 11.5 x 10^{33} Å^6/mole; the quantum yield of fluorescence of the donor is 0.33. The refractive index in the wavelength region of the energy transfer is assumed to be the same value as water which is 1.33; assuming that there are random orientations between the donor and the acceptor, the orientation factor (K^2) has a value of 2/3.

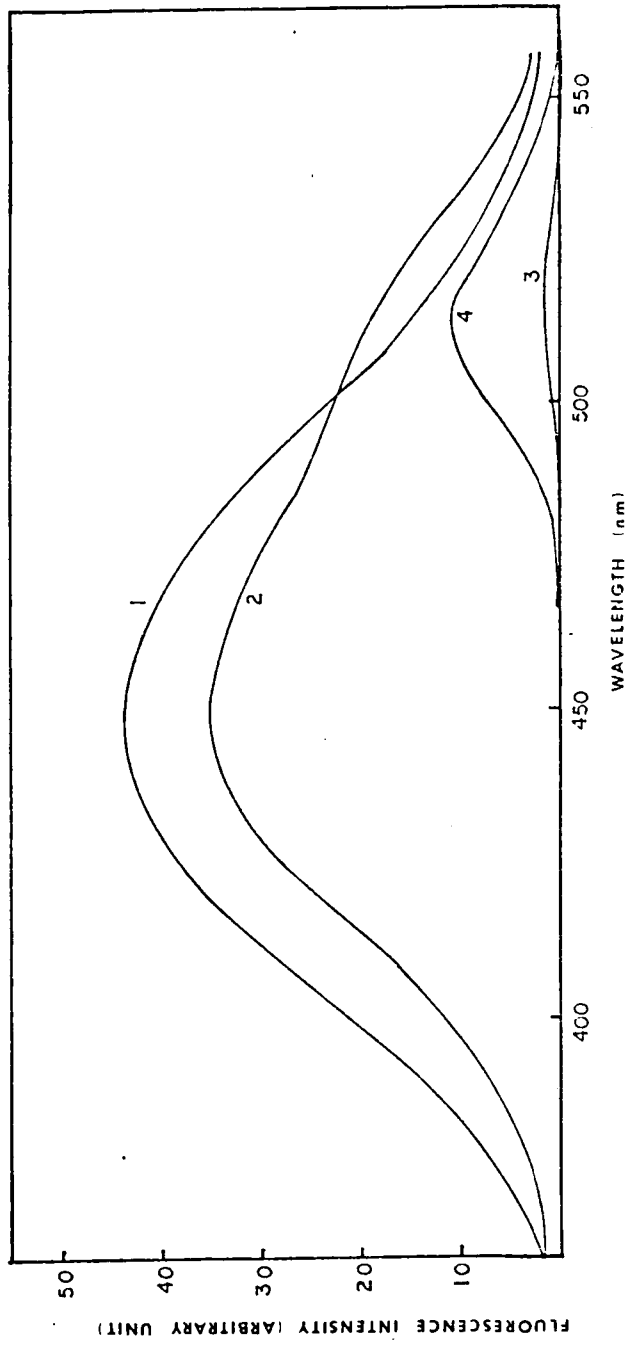

Figure 23. Emission spectra of AEDANS-kinase (1.1μM) (1) and AEDANS-kinase (1.1μM) + IAF-oxidase (1.2μM) (2) excited at 330nm. Emission spectra of IAF-oxidase (1.2μM) excited at 330 (3) and 450nm (4). Experiments conducted in 0.1M potassium phosphate (pH 7).

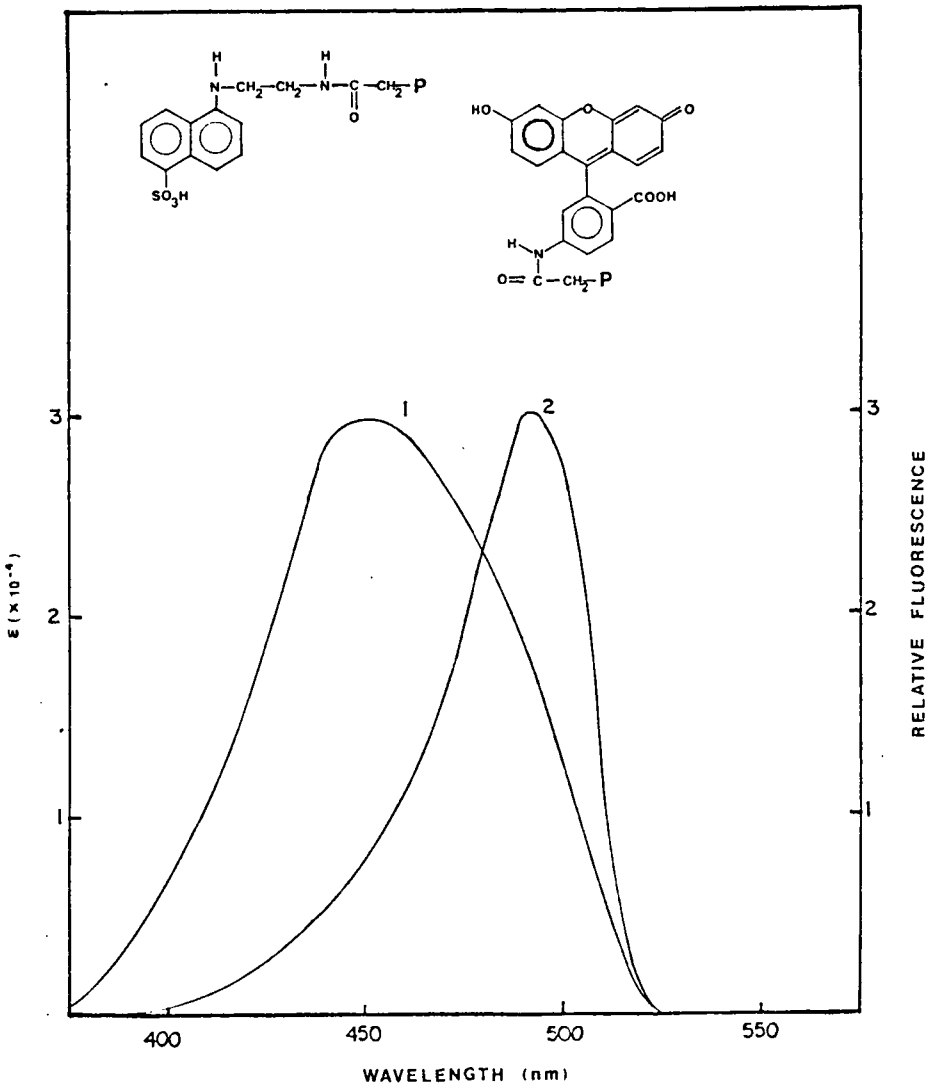

Figure 24. Emission spectrum of pyridoxal kinase (1) labelled with 1 mole of AEDANS per mole of enzyme and excited at 330nm. Absorption spectrum of pyridoxine-5-P oxidase containing 1.2 moles of IAF per mole of enzyme. Experiments conducted in 0.1M potassium phosphate (pH 7). The spectral overlap integral (J) was calculated from the recorded spectra. $J = 11.5 \times 10^{33} A^6$ mole^{-1}.

Using equation 10, the critical distance of energy transfer between the donor and the acceptor was further calculated using equation 11 to be 35Å.

Discussion

The distance between the two fluorophores labelled on pyridoxal kinase and the pyridoxine-P oxidase demonstrated that the two enzymes existed as close pairs in solution because the distance of transfer is shorter than the distance of separation (R = 46Å) between the centers of two adjacent spherical macromolecules characterized by a molecular weight of 60,000. This close distance between the two macromolecules gives support to the idea that the two enzymes may form an aggregate in solution. Further experimental evidence has to be provided in order to prove this contention.

C. POLARIZATION OF FLUORESCENCE

Introduction

Association of interacting macromolecules can be detected by using the technique of polarization of fluorescence. Polarization of fluorescence is measured by monitoring the physical changes in the hydrodynamic properties of macromolecules. This technique is particularly suitable to the study of systems where one of the components is tagged with a fluorescent chromophore. In addition, specific labelling of each component with a fluorescent probe makes it possible to operate at concentrations well below those at which association takes place (58).

Material

The materials used are the same as previously described on page 53.

Method

Polarization of fluorescence measurements was performed the same way as previously described on page 20.

Results

The polarization of fluorescence of iodoacetamide fluorescein labelled pyridoxine-P oxidase (IAF-oxidase) (1.2μM) in the presence of pyridoxal kinase was measured at 25°C. Table VII shows the results of the polarization and fluorescence lifetime measurements. The increase in polarization of fluorescence observed at protein concentrations of 1.2μM were recorded at various excitation wavelengths. As shown by the results in Table VII, the polarization of fluorescence increased from 0.20 to 0.25 at 25°C upon addition of pyridoxal kinase. When the polarization of fluorescence of the mixture containing IAF-oxidase (1.2μM) and pyridoxal kinase (1.0μM) was measured as a function of the temperature, the plot of $(1/p - 1/3)$ versus T/η is linear over the entire range of temperatures examined (Figure 25).

It is pertinent to note that several proteins labelled with fluoresceine-isothiocyanate yield a linear plot of 1/p vs. 1/MW as shown in Figure 26. Furthermore, formation of protein-protein complexes brings about further increase in the polarization of fluorescence of labelled protein. Thus, the addition of concanavalin A to

TABLE VII

FLUORESCENCE PROPERTIES OF AEDANS-KINASE AND IAF-OXIDASE

Sample	Excitation Maximum (nm)	Polarization	Lifetime (m-sec)
AEDANS-Kinase	330	0.08	19.1
IAF-Oxidase	450	0.20	5.1
IAF-Oxidase + Kinase*	450	0.25	5.1
" "	440	0.24	
" "	460	0.25	
" "	470	0.25	

*The concentration of IAF-oxidase and pyridoxal kinase are 1.2 and 1μM, respectively.

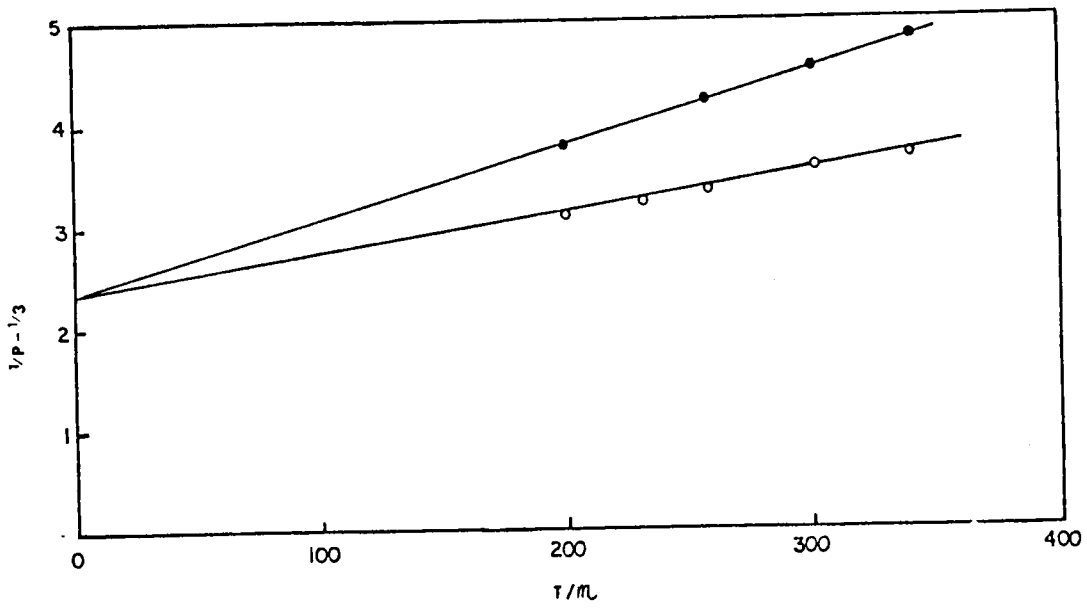

Figure 25. Plots of 1/p - 1/3 vs. T/η for IAF-oxidase (1.2μM) (●) and IAF-oxidase (1.2μM) + pyridoxal kinase (1μM) (○). Excitation wavelength 450nm. A rotational correlation time ratio of 1.6 was obtained at 25°C.

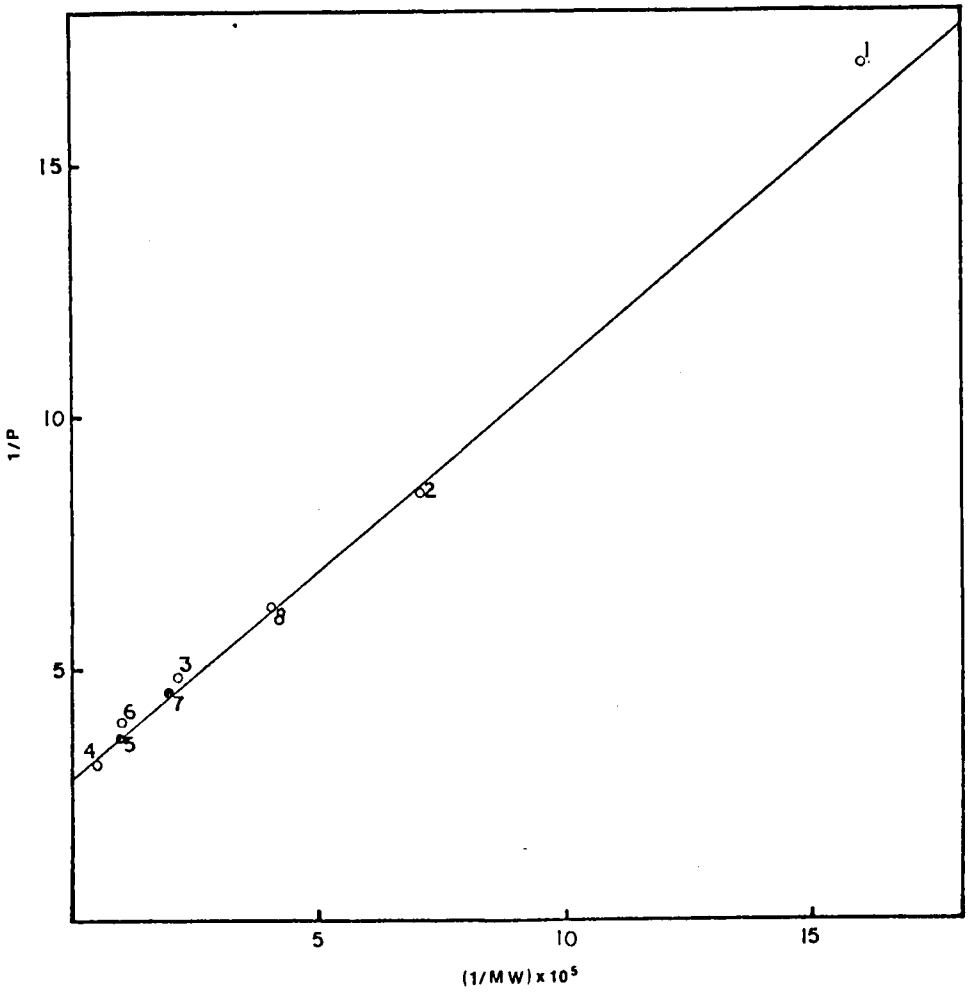

Figure 26. Plot of 1/p versus 1/MW. Samples of proteins were labelled with fluoresceine isothiocyanate (1), pancreatic trypsin inhibitor (2), ribonuclease B (3), ovalbumin (4), immunoglobulin (5), IAF-oxidase (1.2μM) + pyridoxal kinase (1μM) (6), ribonuclease B (10μM) + concanavalin A (5μM) (7), IAF-oxidase (8), pancreatic trypsin inhibitor (2μM) + trypsin (2μM). Experiments conducted in 0.1M potassium phosphate (pH 7) at 25°C. Excitation wavelength 450nm.

labelled ribonuclease B is paralleled by a dramatic increase in fluorescence polarization due to the formation of a protein-protein complex.

The addition of trypsin to labelled pancreatic trypsin inhibitor also results in an increase in the fluorescence polarization and the measured fluorescence polarization corresponds to the molecular weight of the largest macromolecule of the system. This is also true for the system IAF-oxidase + pyridoxal kinase, where the increase in fluorescence polarization is due to the formation of a complex of approximately 110,000 molecular weight.

Discussion

Besides the results obtained from the resonance energy transfer experiment described previously, polarization of fluorescence experiment also provides evidence for the formation of an enzyme-enzyme complex between pyridoxal kinase and pyridoxine-P oxidase. Further experiments on Sephadex gel filtration would confirm the complex formation of the two enzymes.

D. SEPHADEX G-100 GEL FILTRATION

Introduction

Molecular exclusion chromatography is based on the principle of separating molecules by different sizes. Molecules which cannot pass through the Sephadex beads will not be retarded by the gel and will be eluted at the void volume of the elution. If two enzymes form a complex, the total molecular weight of the complex would be larger,

and the elution pattern would be altered when compared with each individual enzyme from the complex. The molecular weight of the complex of pyridoxal kinase and pyridoxine-P oxidase can be evaluated by Sephadex G-100 chromatography.

Materials

Sephadex G-100 was purchased from Pharmacia. Pyridoxal kinase and pyridoxine-P oxidase were isolated together from the procedure described previously. Aspartate aminotransferase isolated from pig brain was a gift from Mr. Udo Moses of this laboratory.

Method

Six mg of protein containing pyridoxine-P oxidase activity and pyridoxal kinase activity were applied to a Sephadex G-100 column (60 x 1cm) and the column was eluted by 0.01M potassium phosphate buffer (pH 6.8) containing 0.1mM glutathione. Forty drops per fraction were collected. The fractions were subjected to pyridoxal kinase and pyridoxine-P oxidase activity assay. Aspartate aminotransferase which has a molecular weight of 110,000 was run in Sephadex G-100 column by the same technique as the pyridoxine-P oxidase and pyridoxal kinase.

Results

Three mg of pyridoxal kinase and 3mg of pyridoxine-P oxidase were mixed together in a volume of 2ml 0.01M potassium phosphate (pH 6.8). The mixture was chromatographed on a column (100 x 0.5cm) of Sephadex G-100. The activity of pyridoxal kinase and pyridoxine-P oxidase were both detected at the same fraction as shown in Figure 27. Aspartate

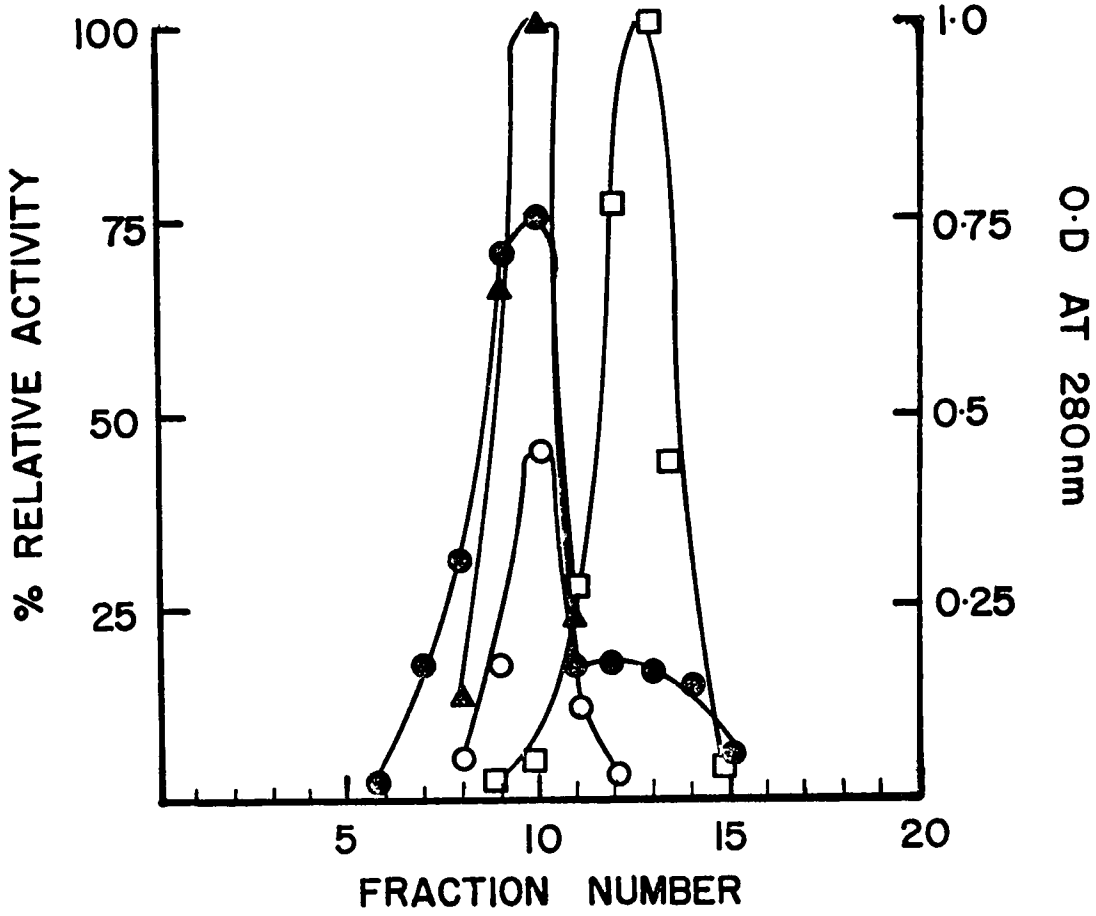

Figure 27. Elution profile of Sephadex G-100 chromatography (100 x 0.5cm). Mixture of (3mg) pyridoxal kinase (●) and (3mg) pyridoxine-P oxidase in a total volume of 2ml (0) was eluted with 0.01M potassium phosphate pH 6.8. The volume of each fraction was 1.8ml. BSA (□) (MW 67,000) and aspartate aminotransferase from pig heart (▲) (MW 110,000) were eluted by the same column individually at identical conditions as the elution of the mixture of pyridoxal kinase and pyridoxine-P oxidase.

aminotransferase from pig brain which has a molecular weight of 110,000 and BSA which has a molecular weight of 68,000 were used as standards for the molecular weight calibration of the column. Aspartate aminotransferase came off the column at the same location as the mixture of pyridoxal kinase and pyridoxine-P oxidase, and BSA came off in later fractions. This shows that pyridoxal kinase and pyridoxine-P oxidase form a complex of molecular weight around 110,000.

Discussion

Complex formation between pyridoxal kinase and pyridoxine-P oxidase was further confirmed by using Sephadex G-100 chromatography. The interaction between the two enzymes results in the formation of a complex of 110,000 MW as determined by Sephadex G-100 gel filtration.

E. SUCROSE DENSITY GRADIENT CENTRIFUGATION

Introduction

Since the enzyme-enzyme complex of pyridoxal kinase and pyridoxine-P oxidase was detected on Sephadex G-100 chromatography, it would be worthwhile to test the stability of this complex in sucrose density gradient centrifugation. If the interaction between the two enzymes persists in sucrose density gradient, then the complex should migrate under the influence of a centrifugal field with a sedimentation coefficient larger than 4.25, the sedimentation coefficient of a globular protein of 60,000 molecular weight.

Material

Sucrose and potassium phosphate (monobasic and dibasic) were purchased from Fisher. Purified lysozyme from egg white and malate dehydrogenase from pig heart were from Boeringer Manheim Biochemicals. Purified lactate dehydrogenase from rabbit muscle and catalase from beef liver were from Sigma Chemical Company.

Methods

The sucrose density gradient was made by 5% and 20% of sucrose in 0.01M potassium phosphate (pH 6.8) in the presence of 0.1mM glutathione and were held in cellulose acetate centrifuge tubes (Beckman) according to the method previously described (page 10). The gradient tubes were installed in a Beckman SW 50.1 rotor and were centrifuged in a Beckman L3-50 centrifuge for 14 hours at 4°C. The fractions were collected by using a peristaltic pump and 26 0.15ml fractions were collected from each tube.

Pyridoxal kinase and pyridoxine-P oxidase activity were assayed according to the method described previously (page 3).

Enzyme standards used in the centrifugation were assayed according to the methods published in The Worthington Manual from Worthington Biochemical Corporation.

Results

After sucrose density gradient centrifugation, pyridoxal kinase and pyridoxine-P oxidase activities were detected in the same fractions collected from the gradient, and the ratio of the two activities

was the same as the sample before it was subjected to centrifugation. This may be due to the fact that both have the same molecular weight. However, the two protein activities were detected in macromolecules heavier than malate dehydrogenase, which has a molecular weight of 70,000. As shown in Figure 28, pyridoxal kinase and pyridoxine-P activities were detected in fractions sedimenting between malate dehydrogenase and lactate dehydrogenase (142,000). By comparison with the standard proteins, the two proteins are found to have a molecular weight of 95,000 and a sedimentation coefficient of 7S.

Discussion

Sucrose density gradient centrifugation has been used to determine the sedimentation coefficient of the two interacting enzymes, i.e. pyridoxal kinase and pyridoxine-5-P oxidase is very strong, the complex would sediment as a macromolecule of 120,000 molecular weight. The results showed that the two enzymes interact strongly and cannot be separated by sucrose density gradients. The complex was found to have a molecular weight of 95,000 which is heavier than the molecular weight of the kinase or the oxidase alone. As a result, the formation of the enzyme-enzyme complex has been firmly established but the affinity between the two enzymes forming the complex remains to be investigated.

F. COUPLING OF PYRIDOXAL KINASE AND PYRIDOXINE-P OXIDASE

Introduction

The formation of pyridoxal-5-P as catalyzed by pyridoxal kinase (PK) and pyridoxine-5-P oxidase (PO) when pyridoxine (PN) and ATP

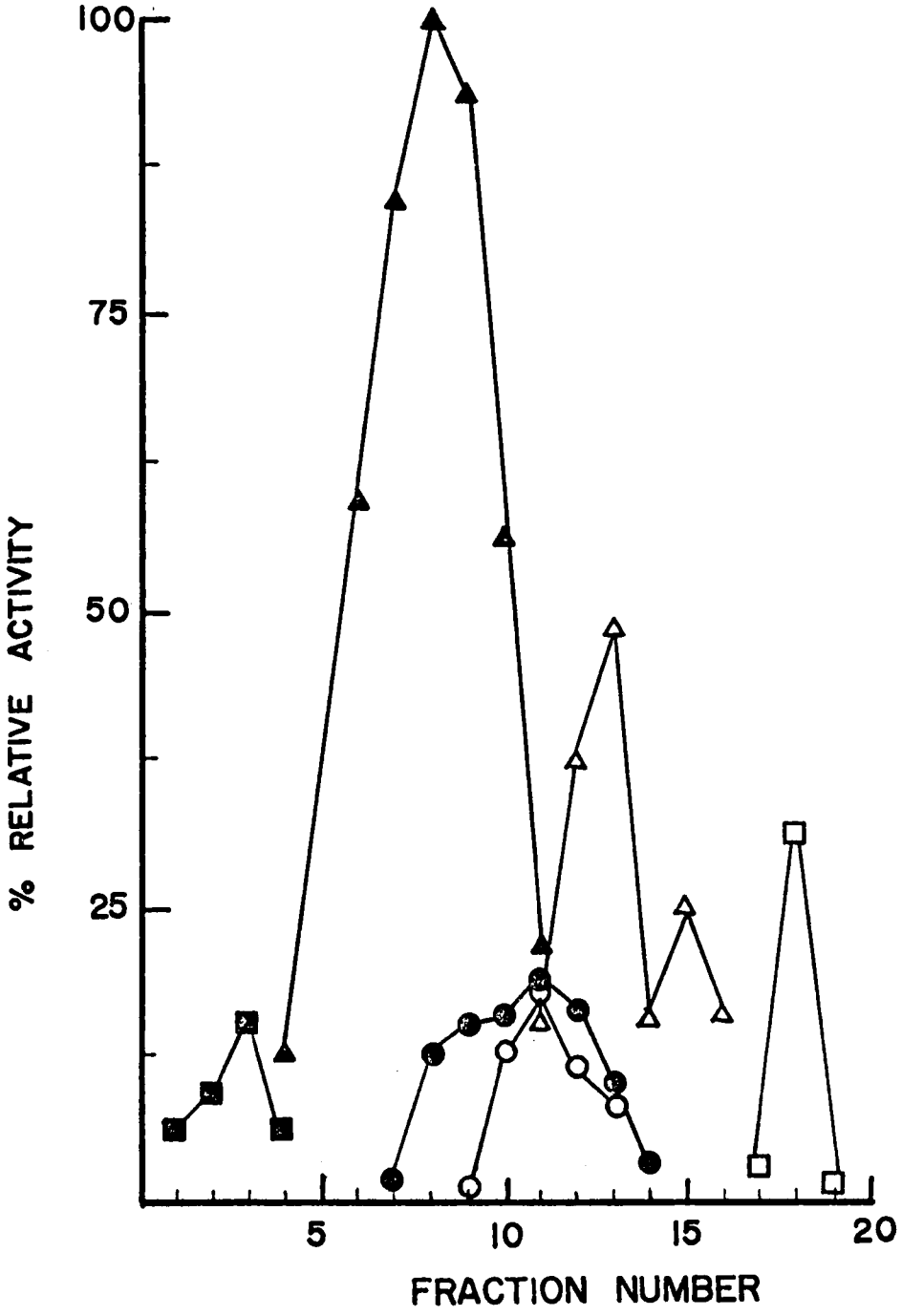

Figure 28. Sucrose density gradient centrifugation of pyridoxal kinase and pyridoxine-P oxidase complex. Molecular weight of the complex was determined by comparing with the molecular weight of standard proteins: [■] catalase MW 240,000, [▲] lactate dehydrogenase MW 142,000, [△] malate dehydrogenase MW 70,000 and lysozyme MW 14,700.[◘][●] pyridoxal kinase and [O] pyridoxine-P oxidase activity were detected together at a position of molecular weight equivalent to 95,000.

are the substrates of the reaction mixture is shown in the following scheme:

$$\begin{array}{lcl} PN & \xrightarrow[k_1]{\text{pyridoxal kinase}} & PNP + PK \\ ATP & & ADP \end{array}$$

$$\begin{array}{lcl} PNP & \xrightarrow{\text{pyridoxine-P oxidase}} & PLP + PO \\ O_2 & & H_2O_2 \end{array}$$

The concentration of pyridoxal-5-P (PLP) formed at any time may be predicted by equation (20)

$$PLP = V \cdot t + \frac{V}{K} \cdot (e^{-k \cdot t} - 1) \qquad (20)$$

provided the first reaction proceeds at constant velocity and the concentration of pyridoxine-5-P [PNP] is low enough for the second consecutive reaction to be first order.

According to the theory developed by Hess and Wurster (38), the transient time (τ) observed in a consecutive reaction is related to the concentration of the second enzyme by equation (21)

$$\tau = \frac{1}{k_2[OX]} = \frac{1}{K} \qquad (21)$$

We have found that when the steady concentration of [PNP] is smaller than the Michaelis constant of the oxidase for PNP, the accumulation of PLP as a function of reaction time followed a pattern similar to that predicted by equation (20).

Material

PNP was prepared by reduction of PLP by sodium borohydride and purified by crystallization.

Methods

Since the two enzymes have different optimum pH, pH 6 for the kinase and pH 8 for the oxidase, pH 7 was chosen to conduct the kinetic experiments of the coupled assay. The buffer used was 0.07M potassium phosphate pH 7.

Kinetic studies were conducted in an Aminco DW-2 spectrophotometer with quartz curvettes of total volume equal to 1ml and path length 1cm.

Results

In a typical experiment, the rate of formation of pyridoxal-P at a concentration of pyridoxal kinase (0.015mg/ml) and pyridoxine-P (0.125mg/ml) displays a transient time of 130 seconds. Similar kinetic measurements conducted at varying concentrations of pyridoxine-P oxidase yielded the results in Figure 29.

The plot of log $1/\tau$ versus log [PO] follows a linear pattern up to concentrations of enzyme of approximately 6μM. At concentrations of oxidase above 6μM, the plot of $1/\tau$ versus log [PO] shows a marked deviation from the linearity expected for a system where there is no interaction between the enzymes participating in catalysis. Thus, the transient times observed at oxidase concentrations of 6, 7, 8 and 9μM are considerably shorter than the values calculated from equation (21).

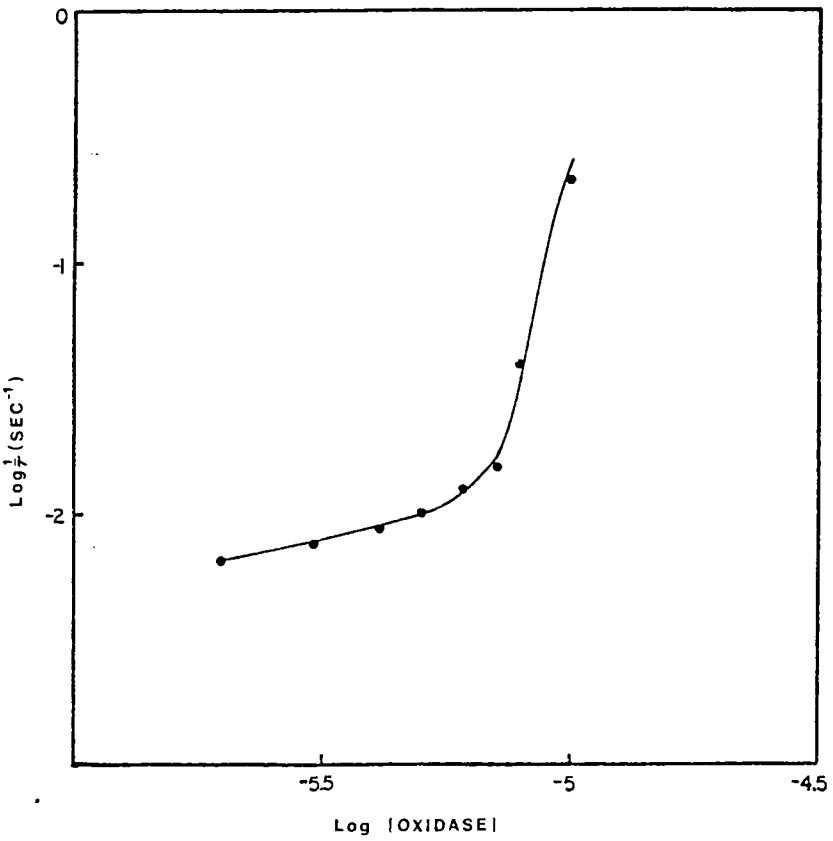

Figure 29. Relation between the reciprocal transient time (τ) and the molarity of pyridoxine-5-P oxidase. Enzymatic assays performed in 0.1M potassium phosphate (pH 7) containing ATP (0.1mM), pyridoxine (0.1mM) and $ZnCl_2$ (0.05mM). The concentration of pyridoxal kinase (0.25μM) was kept constant, whereas pyridoxine-5-P oxidase was varied from 2μM to 9μM.

Discussion

Transient kinetic analysis showed that interaction between pyridoxal kinase and pyridoxine-P oxidase occurred during the conversion of pyridoxine to pyridoxal-5-P. The importance of the interaction was the direct delivery of pyridoxine-P from pyridoxal kinase to pyridoxine-P oxidase so that there was no out-diffusion of intermediates from the consecutive reactions. Although pyridoxal kinase and pyridoxine-P oxidase both have low specific activities for the production of pyridoxal-5-P, this difficulty can be overcome to a certain extent by this complex formation.

G. GENERAL DISCUSSION

Several laboratories have shown that the ability of pyridoxal kinase to phosphorylate pyridoxal is higher than the concentration of pyridoxal-P in different regions of the brain (39). Thus, the kinase alone catalyzes the formation of approximately 200nmol of pyridoxal/1g of tissue per hour, whereas the concentration of pyridoxal-P in different regions of the brain varies between 2 and 4 nmole/1g of tissue.

Since pyridoxine-P oxidase also contributes to the formation of pyridoxal-P, it is evident that some regulatory mechanisms must exist to prevent further formation of pyridoxal-P. The inhibitory effect exerted by pyridoxal-P (K_I = 2.3 x 10^{-5}M) on pyridoxine-P oxidase suggests that the accumulation of pyridoxal-P could, in principle, prevent its further formation from pyridoxine and pyridoxamine. Although

the K_I of pyridoxal-P is still higher than the average concentration of this cofactor in brain, the possibility exists that accumulation of pyridoxal-P is facilitated by macromolecular aggregates which restrict the out diffusion of metabolites.

In this context, it should be noted that a macromolecular aggregate could be formed between the enzyme participating in the catalytic steps leading to formation of the final product.

Our experimental results have shown that the interaction between pyridoxal kinase and pyridoxine-P oxidase reduces the transient time (τ), which can be attributed to such factors as maintenance of high local concentrations of enzymes and restriction of the out-diffusion of intermediates.

Intuitively, one recognizes that the transient time for a multienzyme system must depend on diffusional transit time, in addition to the absolute catalytic properties of the constituent enzymes. According to the molecular model developed by Somogyi and Damjanovich (40), the transient time (τ) for a two step enzymatic reaction can be expressed by a general equation (22)

$$\tau = \frac{1}{R_1 D_1 [E_2]} = e^{-E/K_B T} \qquad (22)$$

which includes the activation energy for the chemical steps (E), the concentration of the second enzyme (2), the diffusion coefficient (D_1) and molecular radius (R_1) of the substrate of the second enzyme. This equation clearly illustrates the effects of protein-protein or protein-matrix interactions on both the activation energy (E) and the diffusion

coefficient of the substrate, which is related to the transport properties (e.g. viscosity) of the microenvironment.

Further support for the contention that pyridoxal kinase and pyridoxine-P oxidase from pig brain tend to form stable macromolecular aggregates in solution, was derived from polarization of fluorescence and resonance energy transfer measurements conducted at protein concentrations of around 1μM. When the polarization of fluorescence properties of IAF-oxidase (1.2μM) in the absence and presence of pyridoxal kinase were examined over the temperature range 10°C - 30°C, it was found that the plots of 1/p - 1/3 versus T/η are linear within experimental error and fit Perrin's equation for spherical macromolecules. Taking the fluorescence lifetime of the chromophore to be 5 nanoseconds, the rotational correlation time of the oxidase in the presence of pyridoxal kinase is 1.6 times greater than the rotational correlation time of the oxidase alone (Figuer 25). The increase in apparent rotational correlation time is attributed to the formation of a stable macromolecular complex.

Finally, the method of singlet energy transfer as applied to the system pyridoxine-P oxidase and pyridoxal kinase also gives results which are consistent with a model which assumes direct physical interaction between the two enzymes.

This method has been previously used to deduce proximity relationship between the catalytic sites of aspartate aminotransferase (41) and to study the distance of separation between the subunits of oligomeric protein structures (42-44).

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LIST OF REFERENCES

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