To the Graduate Council:

I am submitting herewith a thesis written by Sean M. Sullivan entitled "Pyridoxine-5-Phosphate Oxidase: Identification of Isozymes and Synthesis of New Substrates." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Biochemistry.

Jorge E. Churchich, Major Professor

We have read this thesis and recommend its acceptance:

Accepted for the Council:

Vice Chancellor
Graduate Studies and Research

PYRIDOXINE-5-PHOSPHATE OXIDASE: IDENTIFICATION OF ISOZYMES AND SYNTHESIS OF NEW SUBSTRATES

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Sean M. Sullivan

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I would like to express my appreciation to Dr. J. E. Churchich for offering his expert guidance and assistance in my research. I would also like to express my deepest appreciation to my parents, Mr. and Mrs. John V. Sullivan, for strengthening my positive attitude toward my goal through their confidence, kindness and understanding.

In closing, I would like to thank my friends—you know who you are—for making the hard times not so hard and the good times seem that much better.

ABSTRACT

Pyridoxine-5-phosphate oxidase was purified from pig liver. A coupled fluorometric enzymatic assay was developed. The assay offers increased sensitivity and the capability of continuously monitoring the time course of the reaction.

Polyacrylamide gel electrophoresis coupled with activity staining revealed the existence of more than one form of the oxidase. Further analysis revealed that these forms have similar molecular weights but vary in their charge to mass ratios.

Phosphopyridoxyl analogues were used to study properties of the active site. Aliphatic and aromatic carboxylic acids were attached to the primary amino group of pyridoxamine-5-phosphate. The aliphatic carboxylic acids ranged from three to seven methylene groups in length. The active site is capable of accommodating aliphatic carboxylic acids up to five methylene groups in length.

Phosphopyridoxyl-m-aminobenzoic acid yielded a two-fold increase in the k_{cat} and a ten-fold decrease in the K_M in comparison to the aliphatic carboxylic acid analogues. These dramatic changes in the kinetic parameters for phosphopyridoxyl-m-aminobenzoic acid are related to the effect of the aromatic ring on the amine. Alteration of the the electronic configuration may facilitate formation of the transition intermediate. Once formed, the intermediate is capable of being stabilized by the additional resonance energy offered by the aromatic group.

Separation distance between the bound FMN and the substrate binding site of the oxidase was determined by resonance energy transfer experiments. A value of 17 Å was obtained.

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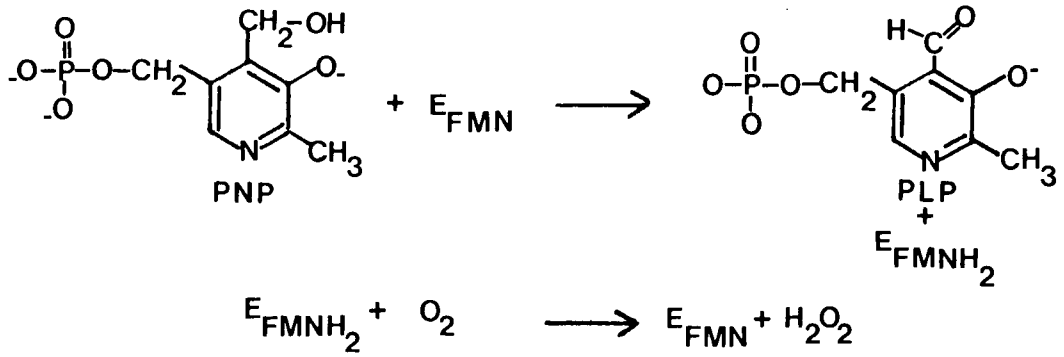
CHAPTER I

INTRODUCTION

Pyridoxine-5-phosphate oxidase (PNP-oxidase, EC-1.4.3.5.) is an enzyme involved in the metabolism of pyridoxal-5-phosphate (PLP). The cytosolic protein has been purified to homogenity from two tissue sources, pig brain (12) and rabbit liver (11). The enzyme has a molecular weight of 56,000 daltons. It is composed of two subunits (MW = 27,000 daltons), plus one molecule of flavin mononucleotide (FMN) bound per enzyme molecule. Oxidase catalyzes the oxidation of PNP to PLP. In the process, the enzyme is reduced. It is regenerated to the oxidized form by an interaction with molecular oxygen. The resulting product is hydrogen peroxide.

The reaction scheme is represented below:



The enzyme also retains the capability of deaminating primary and secondary phosphopyridoxylamines. The resulting products of the enzyme

catalyzed reaction are PLP, free amine and hydrogen peroxide. The mechanism for either type of substrate has not been extensively investigated. The mechanism for deamination does not appear to be as straight forward as the oxidation of the primary alcohol.

PNP oxidase exhibits product inhibition with respect to PLP. The inhibition is competitive with respect to PNP, and the K_I is on the order of 1×10^{-5}M.

Enzymes possessing this characteristic are usually associated with a control point in a metabolic pathway (3). It is for this reason PNP oxidase is a proposed regulatory control point in the metabolism of PLP (11, 12, 20). Product inhibition is not only confined to the studies with purified enzyme. It has been observed in a crude liver homogenate (16), thus characterizing this property as a physiological function. The significance of this observation in the crude homogenate is unclear, for intracellular concentrations of unbound PLP are ten-fold lower than the apparent K_I. A metabolic control model has also been proposed for the brain oxidase (12). It involves an interaction between the oxidase and pyridoxal kinase, another enzyme involved in PLP metabolism.

Pyridoxal kinase phosphorylates pyridoxal, pyridoxine and pyridoxamine at the expense of ATP. Once phosphorylated, PNP and PMP are converted to PLP by the oxidase. A proposed aggregation between the two enzymes results in a localization of a high concentration of PLP at the active site of the oxidase. There is strong evidence supporting a close proximity between the oxidase and the kinase at low concentration (12). The direct effect of the kinase on the catalytic properties of the oxidase has yet to be observed.

The above two theories are currently under investigation to determine the physiological role of product inhibition exerted on PNP oxidase by PLP.

Amino acid modification studies have revealed the existence of two reactive sulfhydryl groups which are not related to catalysis or to binding of the coenzyme. This type of study has also revealed the existence of an essential histidine (10). This histidine is not associated with the binding of FMN to the apo-oxidase.

Phosphopyridoxyl analogues have revealed substrate substituent group requirements necessary for catalysis (11, 12). A dianionic group attached to the 5 α-position and a hydroxyl group attached to the 3 position of the pyridine ring are required for substrate binding. Secondary amines attached to the 4 α-position display enzymatic activity. The nature of the amine governs the K_m and V_{max}.

Analogues of FMN have revealed the requirements for binding and activity of the coenzyme (17). The ribityl-5-phosphate group attached at N-10 is intimately associated with apo-protein groups. The faces of 7,8-dimethyl isoalloxazine ring are in contact with at least one aromatic amino acid. The pyridiminoid edge of the isoalloxazine ring is in contact with polar protein groups. The dimethylbenzene edge is in contact with the solvent. Indirect evidence indicates that the redox potential of the flavin is governed by N-1 and O-2. There is no direct evidence pertaining to the mode of action by which the flavin participates in catalysis.

This is a summary of all the characteristics presently known for the oxidase. There are still many unanswered questions pertaining to mode of

action, conformation, bisubstrate kinetics, and so forth. The research to be presented further characterizes PNP oxidase.

CHAPTER II

FLUOROMETRIC ASSAY

Materials and Methods

Instrumentation

Fluorescence measurements were made using a Bausch & Lomb dual monochromator fluorimeter. A mercury-xenon lamp was used as an excitation light source. Fluorescence emission was monitored directly from a Pacific photometric recording photometer, or transferred from the photometer to a Mosley-Autograf X-Y recorder. The fluorimeter cuvette chamber accommodated a 3 ml cuvette.

Enzymatic Assays

A 2 ml reaction mixture contained 1×10^{-4}M pyridoxine-5-phosphate, 0.05 M amino-oxy acetic acid in 0.1 M potassium phosphate buffer, pH 8.0. The reaction was initiated by the addition of pyridoxine-5-phosphate oxidase. The reaction mixture was excited at 380 nm and fluorescence emission was monitored at 460 nm.

Standard for Fluorescence Calibration

A series of 5—2 ml solutions—were prepared containing 0.025 M amino-oxy acetic acid, 1×10^{-6}M flavin mononucleotide in 0.1 M potassium phosphate buffer, pH 8.0. Pyridoxal-5-phosphate was added to each tube. The concentration ranged from 5×10^{-7}M to 5×10^{-6}M. Each standard was excited at 380 nm and fluorescence emission was monitored

at 460 nm. A graph of relative fluorescence versus pyridoxal-5-phosphate yielded a straight line.

Determination of the Bimolecular Rate Constant for Formation of Phosphopyridoxyl-Amino-Oxy Acetic Acid

The rate constant for the formation of phosphopyridoxal-amino-oxy acetic acid was calculated under pseudo-first order conditions. Pyridoxal-5-phosphate concentration, 1×10^{-4}M, was held constant. Varying concentrations of amino-oxy acetic acid were added to pyridoxal-5-phosphate such that amino-oxy acetic acid was in 10- to 100-fold excess of PLP. Amino-oxy acetic acid concentrations were varied from 1×10^{-3}M to 5×10^{-2}M. The course of the reaction was monitored by the decrease in absorbance at 388 nm (PLP λ_{max} = 388 nm, $\varepsilon = 4.9 \times 10^{3}M^{-1}cm^{-1}$).

The rate constant was determined using the following equations (8):

For the reaction

$$PLP + AOA \underset{k_{-1}}{\overset{k_{+2}}{\rightleftharpoons}} PLP\text{-}AOA \qquad (II.1)$$

$$\frac{dC_{PLP\text{-}AOA}}{dt} = k_{+2} C_{PLP} C_{AOA} - k_{-1} C_{PLP\text{-}AOA} \qquad (II.2)$$

If $k_{+2} >> k_{-1}$, then

$$\frac{dC_{PLP\text{-}AOA}}{dt} = k_{+2} C_{PLP} C_{AOA} \qquad (II.3)$$

If $k_{obs} = k_{+2}C_{AOA}$, and $C_{AOA} >> C_{PLP}$, then

$$\frac{dC_{PLP\text{-}AOA}}{dt} = K_{obs}\, C_{PLP} \qquad (II.4)$$

$$\frac{dC_{PLP\text{-}AOA}}{} = -\frac{dC_{PLP}}{dt} \qquad (II.5)$$

$$\frac{-\, dC_{PLP}}{dt} = K_{obs}\, C_{PLP} \qquad (II.6)$$

$$-\int \frac{dC_{PLP}}{C_{PLP}} = \int K_{obs} \cdot dt \qquad (II.7)$$

$$\ln \frac{C^{o}_{PLP}}{C_{PLP}} = K_{obs} \cdot t \qquad (II.8)$$

A graph of $\ln \frac{C^{o}_{PLP}}{C_{PLP}}$ versus t yielded a linear plot with a slope equal to K_{obs}; a K_{obs} was computed for each concentration of amino-oxy acetic acid. A secondary plot was obtained using the following equation:

$$K_{obs} = k_{+2}\, C_{AOA} + K_{-1} \qquad (II.9)$$

The K_{obs} was graphed against the corresponding concentration of amino-oxy acetic acid resulting in a slope equal to the forward bimolecular rate constant, k_{+2} ($sec^{-1}M^{-1}$), and the Y intercept was equal to the reverse first order rate constant, k_{-1} (sec^{-1}).

Apparent K_I Determination of PLP-AOA for Pyridoxine-5-Phosphate Oxidase

A 1-ml reaction mixture was prepared in a 1.5 ml fluorescence cuvette containing 0.1 mM PNP in 0.1 M potassium phosphate buffer, pH 8.0. The reaction was initiated by addition of oxidase to the reaction mixture and enzymatic activity was monitored by increase in absorbance at 388 nm using an expanded scale spectrophotometer. Enzymatic activity was monitored in the presence of varying concentrations of phosphopyridoxyl-amino-oxy acetic acid. The apparent K_I was determined using the following equation:

$$v_o = v_I + v_I \ (I/K_I) \qquad \text{(II.10)}$$

$$v_o/v_I = (1 + I/K_I) \qquad \text{(II.11)}$$

where v_o, v_I, I, and K_I represent the reaction velocity in the absence of inhibitor, reaction velocity in the presence of inhibitor, the inhibitor concentration, and the dissociation constant for enzyme-inhibitor complex, respectively. A graph of v_o/v_I versus I yielded a slope equal to $1/K_I$.

Phosphopyridoxyl-amino-oxy acetic acid was donated by Dr. Udo Moses Ekanemesang.

Results and Discussion

The fluorometric enzyme assay is a coupled assay. PNP oxidase oxidizes PNP to form PLP. The resulting product reacts with amino-oxy acetic acid to form phosphopyridoxyl-amino-oxy acetic acid. The

substrate, PNP, is a fluorescent compound. The emission maximum was 400 nm and the excitation maximum was 340 nm. Phosphopyridoxyl-amino-oxy acetic acid has an emission maximum at 460 nm and two excitation maxima at 340 nm and 360 nm. PNP was in much excess compared to the concentration of PLP. Therefore, an excitation maximum was selected which would minimize the background fluorescence contributed by the substrate. This was achieved by using 380 nm as the excitation wavelength. The decrease in background increased the sensitivity of the assay.

The assay involved the modification of product, PLP, to yield the fluorescent compound, phosphopyridoxyl-amino-oxy acetic acid. Oxidase exhibits product inhibition. To ensure that product modification did not increase the degree of inhibition, an apparent K_I was determined with the assumption that competitive inhibition was maintained.

The data are illustrated in Figure II.1. The graph v_o/v_I versus I, yielded a linear plot. The slope was equal to $1/K_I$. The apparent K_I was 4.4×10^{-5}M. This did not deviate significantly from the K_I for PLP ($K_I = 2 \times 10^{-5}$M). Therefore, modification of the product did not increase inhibition.

A coupled assay to be reliable must represent the true rate of enzymatic catalysis. Therefore, the rate-limiting step must be shown to exist in the enzyme-catalyzed reaction and not with the rate of product modification. This was achieved by correlating the rate of product formation observed spectrophotometrically with the rate observed spectrofluorometrically. Figure II.2 represents a reaction time course obtained utilizing the spectrofluorometric assay. The rate was linear

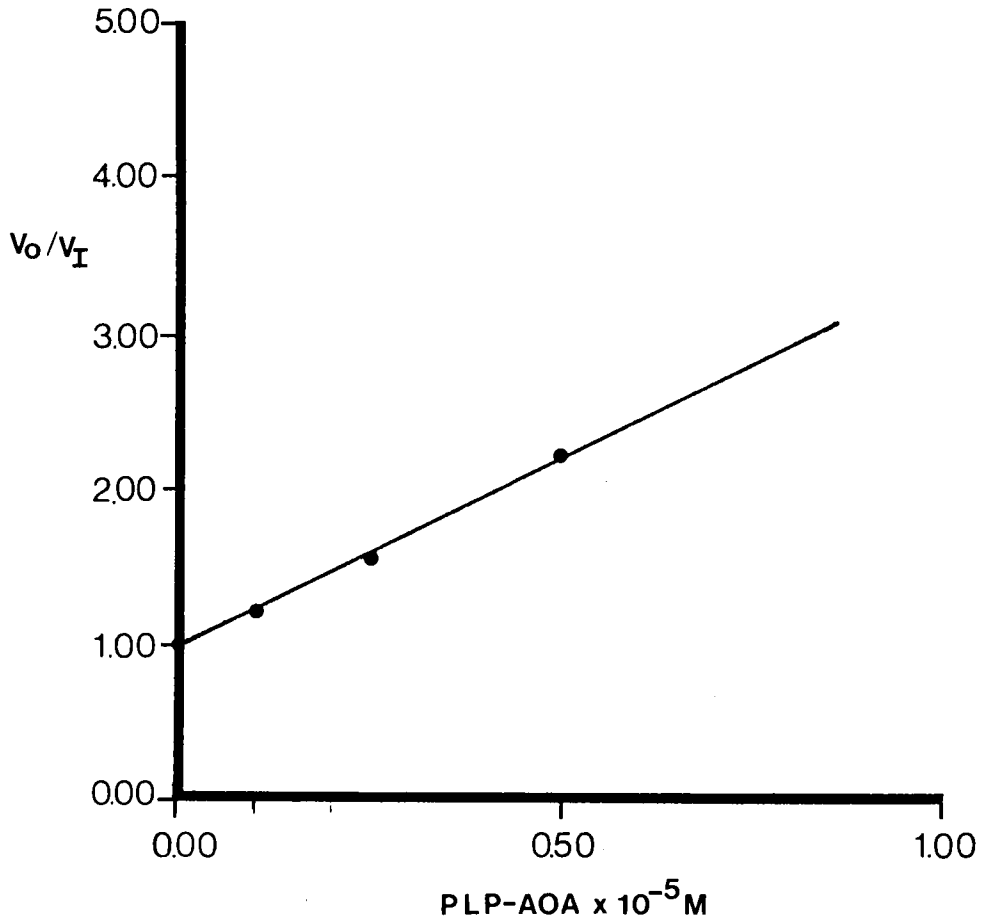


Figure II.1. Determination of apparent K_I for PLP-AOA with respect to PNP. The assay mixture consisted of 0.1 mM PNP, 0.05 mg PNP oxidase, and varying concentrations of PLP-AOA in 0.1 M potassium phosphate buffer, pH 8.0.

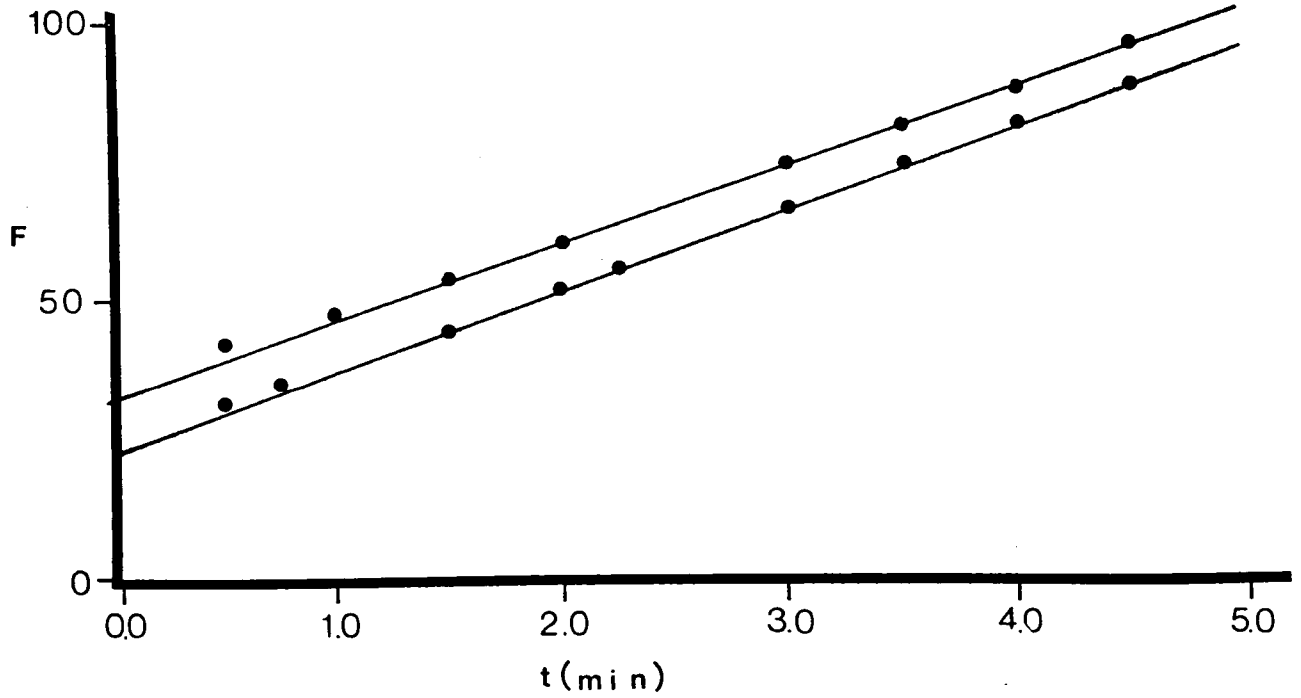


Figure II.2. Fluorometric enzyme assay for PNP oxidase. The assay mixture contained 0.1 mM PNP, 0.05 M AOA, 0.10 mg of PNP oxidase. Assay mixture was excited at 380 nm and fluorescence was monitored at 450 nm as a function of time.

for five minutes. The fluorescence scale was calibrated using a standard plot and a value of 4.1 nm/min/ml of enzyme was obtained. The rate agrees very well with the rate obtained spectrophotometrically (v = 4.1 nm/min/ml of enzyme).

To further strengthen the conclusion, a second order rate constant for product modification was determined. The data are represented in Figures II.3 and II.4 and analyzed as described in the Methods section. A value of 0.4 $M^{-1}sec^{-1}$ was obtained for the forward rate and a value of $1.45 \times 10^{-3}sec^{-1}$ was obtained for the rate of dissociation of final product. These data confirm the initial assumption that the rate of dissociation was negligible. Because the dissociation rate was negligible, the fluorescence emission was representative of the total product formed. Hence, the spectrofluorometric oxidase assay yielded a true representation of the enzymatic rate of product formation.

The fluorometric assay offers several advantages over preexisting assay procedures. Product formation can be monitored continuously as opposed to a time lapse assay. Because it is a fluorometric assay, low concentrations of PLP can be monitored (PLP = $1 \times 10^{-7}M$). The requirement for sophisticated instrumentation is eliminated, such as an expanded scale spectrophotometer.

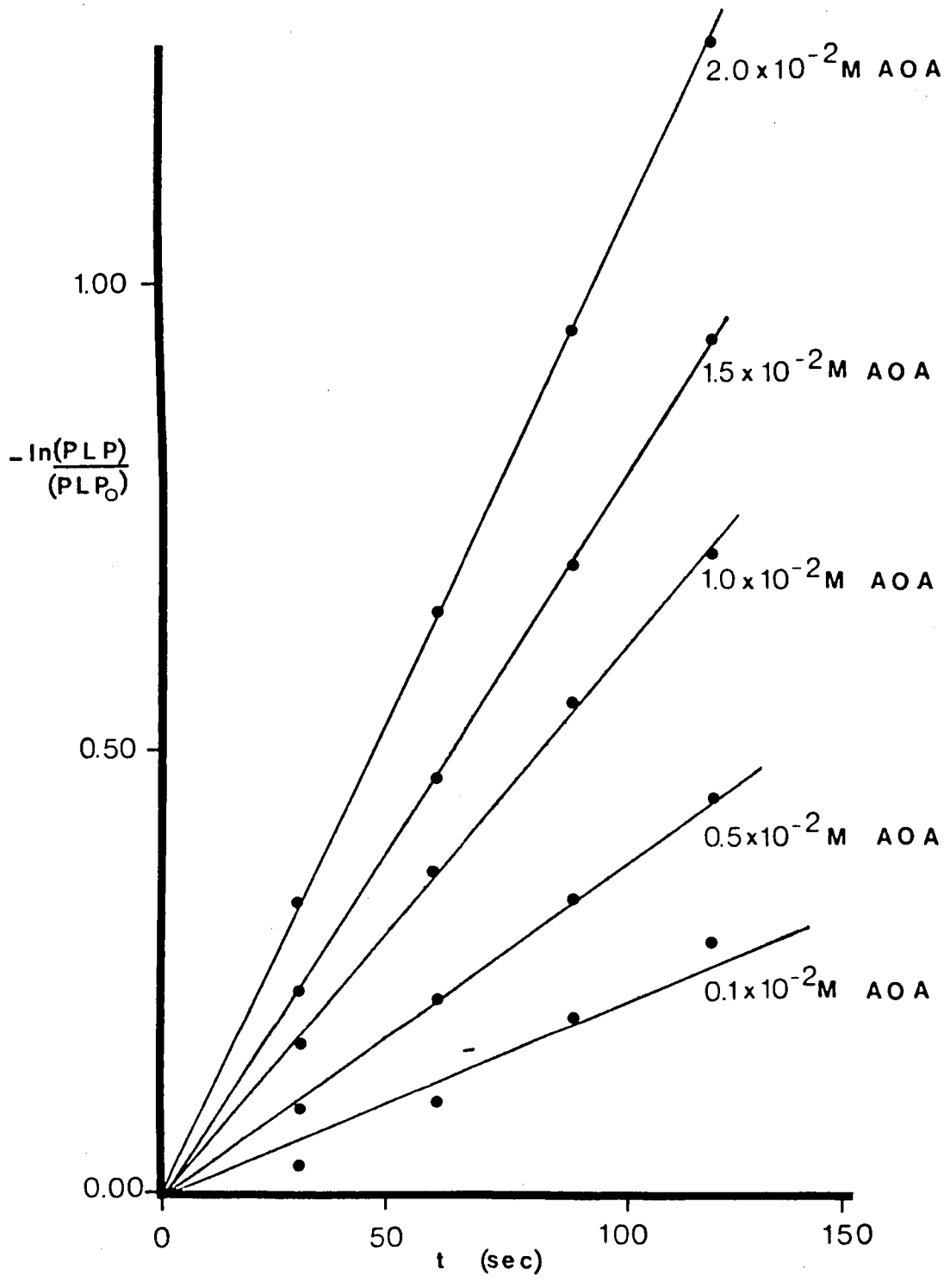


Figure II.3. Determination of the pseudo first order rate constants for PLP-AOA formation. PLP, 0.1 nM was incubated with concentrations of AOA ranging from 1 to 20 mM in potassium phosphate buffer, pH 8.0. A K_{obs} was obtained for each concentration of AOA.

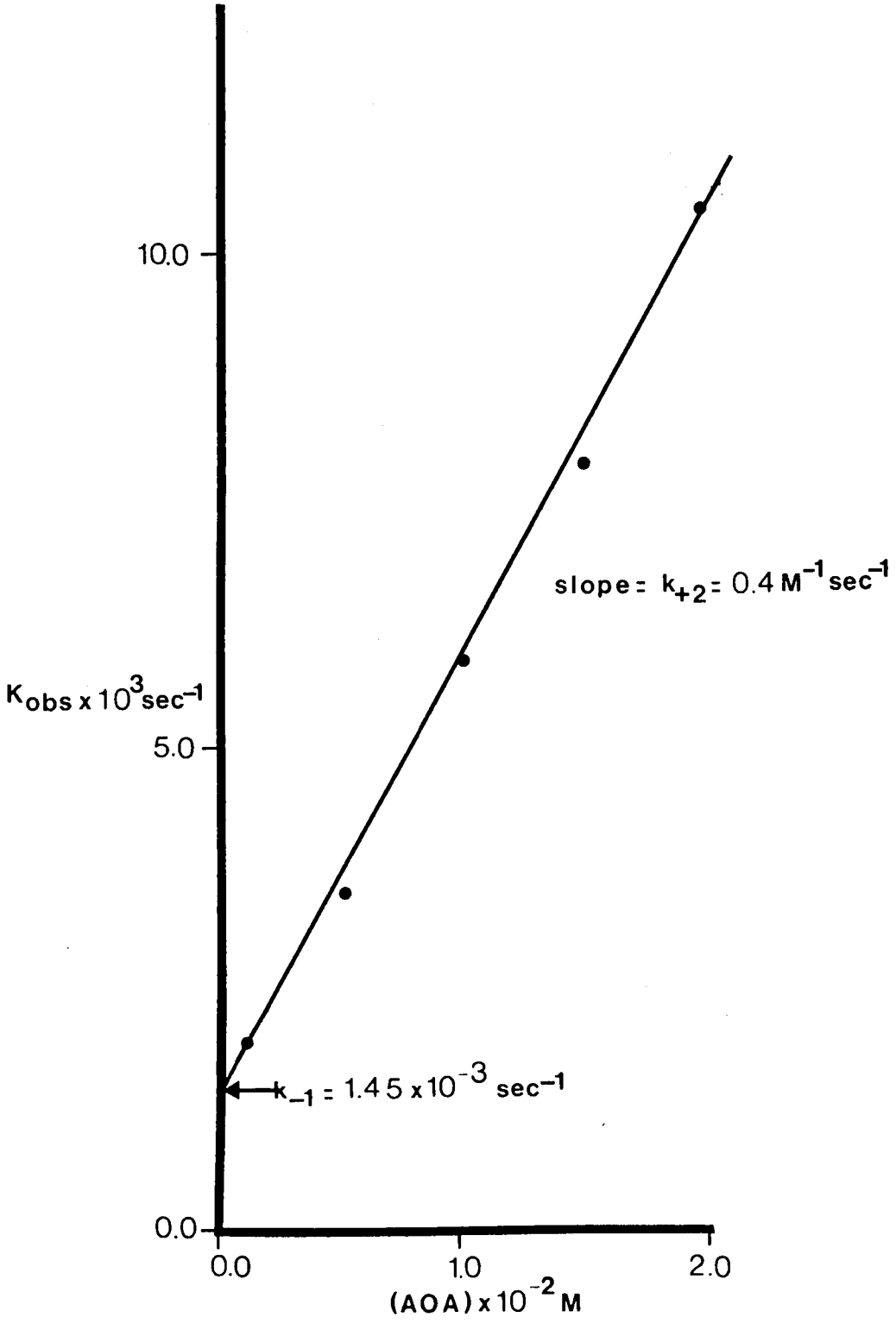


Figure II.4. Secondary plot for the determination of the second order rate constant for the formation of PLP-AOA. The data for this figure were obtained from Figure II.3.

CHAPTER III

PURIFICATION OF PYRIDOXINE-5-PHOSPHATE OXIDASE FROM LIVER AND PRELIMINARY CHARACTERIZATION OF ISOZYMES

Materials and Methods

Isolation Procedure-All purification steps were carried out at 4°C. Pig liver was obtained from East Tennessee Packing Co., and preparation was initiated the same day. One kilogram of liver was homogenized in 4 L of 0.075 M potassium phosphate buffer, pH 6.8. Homogenate was centrifuged at 10,000 rpm for 30 minutes in a Beckman J-10 rotor. Supernatant was pooled and the pH was adjusted to 5.0 with 2 N acetic acid. After stirring for 10 minutes under the conditions described above, the solution was centrifuged and the pH of the supernatant was readjusted to pH 7 using 2 M sodium hydroxide. Readjustment of pH was followed by additional centrifugation.

To the supernatant was added 20.9 grams of ammonium sulfate per 100 ml of supernatant and allowed to stir for 1 hour. The solution was centrifuged for 30 minutes. To the supernatant was added an additional 9.4 grams of ammonium sulfate per 100 ml of supernatant and allowed to stir for 1 hour. The solution was centrifuged for 30 minutes. The pellets were collected, and resuspended in 100 ml of 0.1 M potassium phosphate buffer, pH 8.0 (buffer A). The resuspended pellet was dialyzed against 120 volumes of buffer A for 12 hours.

A 5 × 20 cm DEAE-Sephadex A-50 column was prepared. Twenty grams of anion-exchange resin was swelled in 3 L of water for 2 hours at 90°C. The gel was batch equilibrated with buffer A. The column was poured and packed by flow with 2 column volumes of buffer A. Enzyme was eluted using a 1 L gradient ranging from 0.1 M to 0.2 M potassium phosphate buffer, pH 8.0. Twenty ml fractions were collected. Enzymatic activity and protein concentrations were determined for each fraction. Those with the highest specific activity were pooled.

The pooled fractions were concentrated to a volume of 10 ml using an Amicon ultrafiltration apparatus equipped with a PM-30 filter. The 10 ml concentrate was dialyzed against 100 volumes of 0.01 M potassium phosphate buffer, pH 6.8 (buffer B). Twenty grams of Sephadex-G-100 superfine was swelled in water for 5 hours at 90°C. The gel was batch equilibrated with buffer B. The column was poured and packed by flow with 3 column volumes of buffer B. Following sample application, 5 ml fractions were collected. Enzymatic activity and protein concentrations were determined for each fraction. Those with the highest specific activity were pooled.

A 1.5 × 10 cm column of hydroxylapatite was prepared. Hydroxylapatite was prepared by the method of Tiselius (19). The column was equilibrated with buffer B. The pooled G-100 fractions were applied and the column was washed with three column volumes of buffer B. Elution was achieved by using a step gradient of potassium phosphate buffer, pH 6.8, with increasing concentration increments of 0.05 M. PNP oxidase was eluted at 0.05 M.

Enzymatic Assays

Three methods for assaying enzymatic activity were employed. Phenylhydrazine assay (20) was a time lapse assay. A 2 ml reaction mixture was prepared containing 0.1 mM PNP, and 0.1 ml of enzyme preparation in 0.1 M potassium phosphate buffer, pH 8.0. The reaction was incubated for 30 minutes at 37°C. The reaction was terminated by the addition of 0.1 ml 100% trichloroacetic acid and the precipitated protein was removed by centrifugation using a desk-top centrifuge. To 0.9 ml of supernatant was added 0.1 ml of phenylhydrazine reagent (2 grams/100 ml 10 N H_2SO_4). Absorbance was read at 410 nm and the concentration of product was determined using the extinction coefficient of 23,000 $M^{-1}cm^{-1}$.

The other two enzyme assays used were the spectrophotometric assay and the spectrofluorometric assay. The methods for each are described in Section III.

Electrophoresis

Native gels were prepared by two methods. The routine 7.5% polymerized gel was prepared according to Davis (4). Varying percent polyacrylamide gels were prepared using the method of Weber and Osborne (22). Isoelectricfocusing was carried out using pre-prepared LKB isoelectric focusing gels. The samples were focused according to the protocol accompanying the gels. Protein visualization was achieved using Coomasie brilliant blue. Oxidase activity was visualized in the gel by activity staining (5). The gels were incubated in 0.1 M potassium phosphate buffer, pH 8.0, containing 1×10^{-4}M PNP, plus 0.1 mg/ml

nitroblue tetrazolium. The gels were incubated at 37°C for times ranging from 1.5 to 6.0 hours, depending on the enzymatic activity of the sample. The oxidase location was visualized by the appearance of a violet-blue band.

Sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis was prepared according to Osborn and Weber (22). A 7.5% SDS gel was prepared containing 0.1% SDS. Bovine serum albumin, ovalbumin and chmotrypsinogen were used as molecular weight markers.

Results and Discussion

The purification of PNP oxidase is summarized in Table I. The final purification step, HAP chromatography, yielded an enzyme that was purified 2,200 fold. Oxidase was obtained in holo form. The absorption spectra is shown in Figure III.1. The 280nm/380nm and 280nm/445nm absorbance ratios were identical with the corresponding ratios for reconstituted liver oxidase (11). Homogeneity was analyzed by SDS gel electrophoresis. Protein visualization yielded one major low molecular weight band (26,000 daltons) and a minor contaminant of large molecular weight (65,000 daltons). A 7.5% nondenaturing gel revealed three major bands and one minor band upon protein visualization. Activity stain revealed two major bands and one faint band coincident with the three major protein bands. Incubation of the gels in activity stain solution in the absence and presence of FMN yielded the same activity stain pattern. This eliminated the possibility of the coexistence of apo and holo forms of the oxidase. This observation was consistent for several preparations of the oxidase. The activity banding pattern was first

TABLE I

SUMMARY OF PURIFICATION OF PYRIDOXINE-5-PHOSPHATE OXIDASE

Treatment	Volume (ml)	Protein (mg/ml)	Activity (U/ml)	Specific Activity (U/mg)
Homogenate	4,000	25.8	1.2	0.05
Acid Precipitation	3,660	14.0	1.3	0.09
35-50 $(NH_4)_2SO_4$ Fraction	300	58.0	10.0	0.17
DEAE-Sephadex Fraction	430	4.4	5.2	1.18
Sephadex G-100 Fraction	125	0.42	11.7	27.86
Hydroxylapatite Fraction	4.0	1.03	119.9	116.41

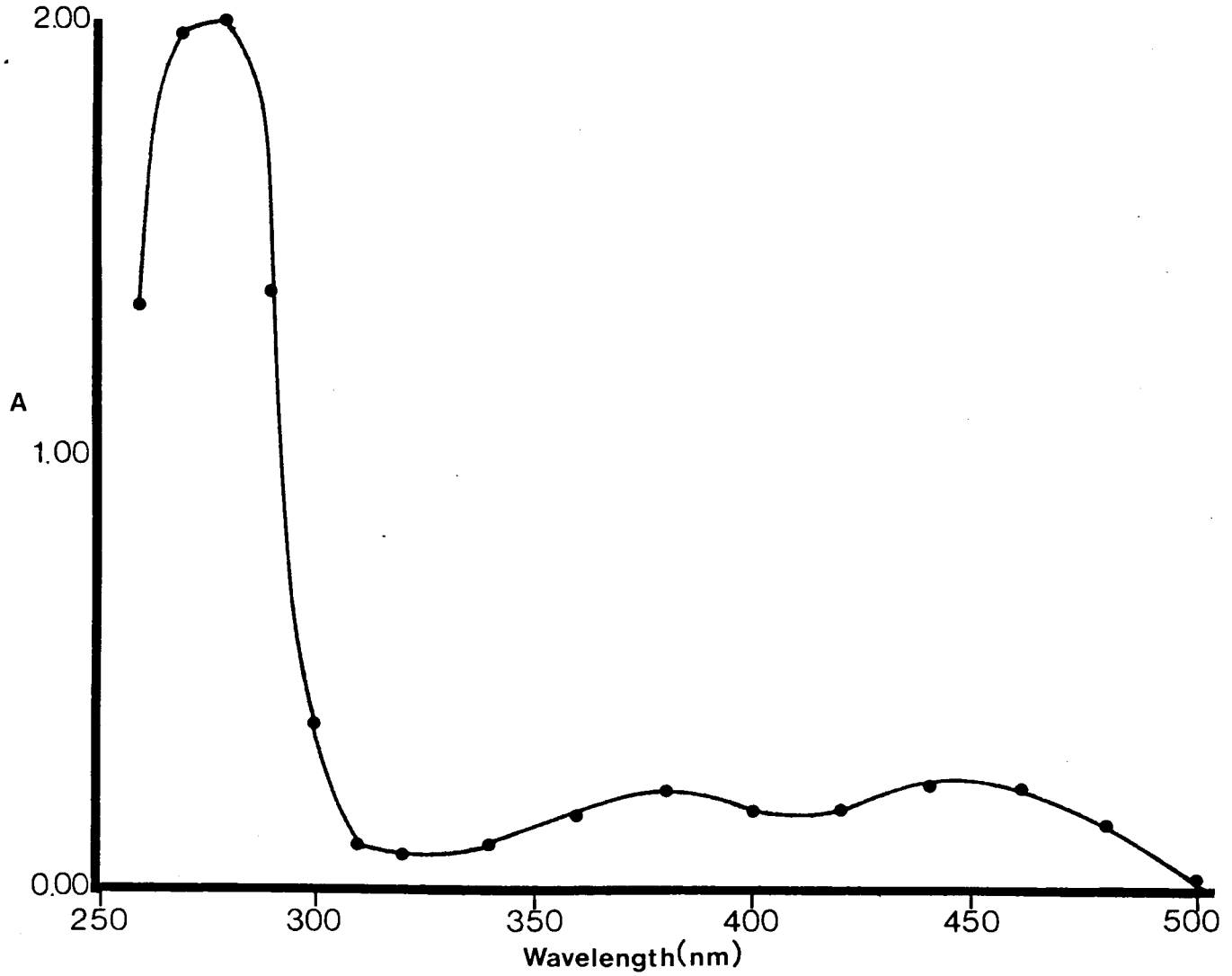


Figure III.1. Absorption spectra of PNP oxidase. The absorption spectra were obtained using an oxidase concentration of 2.2×10^{-5} M in 0.05 M potassium phosphate buffer, pH 6.8.

examined after ion-exchange chromatography step. PNP oxidase from pig brain was isolated by a previously described procedure (12). Visualization of the brain oxidase in 7.5% nondenaturing gel was achieved by activity stain. Two activity bands were observed yielding the same pattern as that obtained from the liver oxidase. The brain oxidase banding pattern was observed after ammonium sulfate fractionation and remained consistent after subsequent isolation procedures.

The difference between the two sources of oxidase was in the band intensities. The brain oxidase yielded two activity bands. One band was of strong intensity and the other was of weak intensity. The two bands of the brain oxidase coincided with the two major activity bands observed with the liver oxidase.

Preliminary classification of the two activity bands observed with the liver oxidase was achieved by maximizing the use of electrophoresis and activity staining. A set of gels was prepared and the percent polyacrylamide was varied (5%, 7.5%, and 9%). The enzyme was electrophoresed and stained for activity. The migration distance was graphed against the percent of polyacrylamide. The data obtained are illustrated in Figure III.2.

A difference in molecular weight between the two bands would result in intersecting lines. The slopes for both lines are identical, confirming that they are parallel. Therefore, the molecular weight of each band is similar, but there is a difference in the charge to mass ratio.

Isoelectric focusing gels with a pH range of 3 to 9 were run and stained for protein and activity. Protein staining revealed three

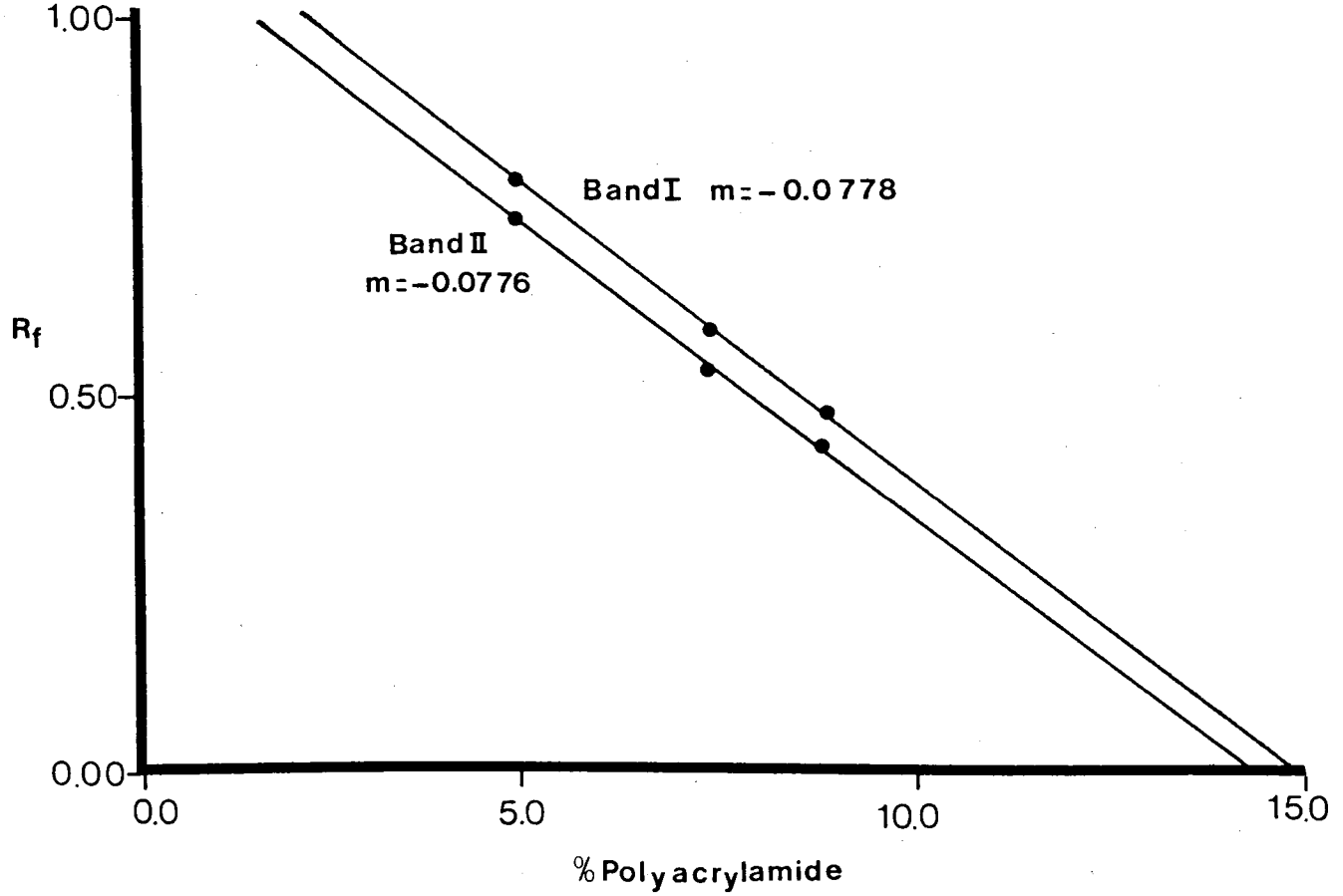


Figure III.2. Determination of migration distance versus percent polyacrylamide concentration for PNP oxidase. The ratio of acrylamide to N,N'-bisacrylamide was held constant. Oxidase was visualized by activity staining. The R_f value was obtained by dividing the migration distance of the activity band by the migration distance of the tracking dye.

distinct bands, two of strong and one of weak intensities. The isoelectric pH was determined for the two major bands, 6.2 and 6.4.

Separation of these oxidase forms was attempted, using DEAE-Sephadex A-50 and also DEAE-cellulose. Several conditions of varying ionic strength and pH were used. The column would retain the enzyme. Upon elution with a linear gradient using concentrations of phosphate buffer ranging from the equilibration buffer concentration to 0.2 M, a single broad activity and protein profile resulted. Gel electrophoresis of fractions from different areas of the broad peak, upon activity staining, revealed a major band accompanied by minor contamination of the other forms. Therefore, homogeneous separation of the isoenzymes could not be achieved using these techniques.

The results obtained from the previous experiments are summarized in the following discussion. The purification scheme employed for isolation of PNP oxidase offers three advantages compared to preexisting isolation schemes (11, 12). The procedure eliminates harsh treatment of the enzyme, such as ethanol precipitation, heat denaturation, repeated lyophilizations, and complete resolution of the enzyme. The enzyme obtained is very stable and it is obtained in the holo form. The enzyme can be purified in five days, whereas preexisting procedures require two weeks.

Classification of the multiple forms of the oxidase was carried out on a preliminary basis. Initially, these forms were observed by staining a nondenaturing gel for oxidase activity. Two bands of equal intensity were observed. This observation was consistent with several different oxidase isolations. Electrophoresis of the oxidase on

polyacrylamide gels of varying percent polyacrylamide has shown that the distance separating the two activities is conserved. Electrophoretic migration is based upon molecular size and charge to mass ratio. This experiment suggests that the molecular weights are similar, but a difference in the charge to mass ratio exists between the forms of the oxidase. This conclusion is further substantiated by the results obtained from SDS-gel electrophoresis. A single molecular weight band is observed. A difference in molecular weight would result in additional low molecular weight bands. The isolation procedure yields holo oxidase. Therefore, these forms of the oxidase are not the result of reconstitution of resolved apo oxidase.

Further evidence supporting the charge to mass ratio difference is offered by isoelectric focusing results. The two major bands were separated by a difference of 0.2 pH units.

Hypoxanthine-Guanine Phosphoribosyltransferase (HPRT) from human erythrocytes (18) exists in three forms. These three forms are separated initially by preparative isoelectric focusing. The three forms have pIs 5.65, 5.80, and 6.01. After isolation, each form is subjected to electrophoresis in a nondenaturing gel. The enzyme with the highest pI migrates the least. The three forms have been shown to have similar molecular weights.

This type of analysis for altered enzyme forms has been applied to a more characterized system—hemoglobin in the normal and sickel form (21). The two forms of hemoglobin are of identical molecular weight, but differ in charge, which is associated with two glutamyl residues. The two forms can be separated on nondenaturing gels illustrating the

difference in the charge to mass ratio. Normal hemoglobin has a pI of 6.9 and sickeled hemoglobin has a pI of 7.1.

These two examples illustrate that separation of isozymes of equal molecular weight can be achieved on the basis of charge to mass ratio. Accompanying the difference in charge to mass ratio is a 0.2 to 0.35 pH unit separation of the pI values for the isozymes of hemoglobin and HPRT.

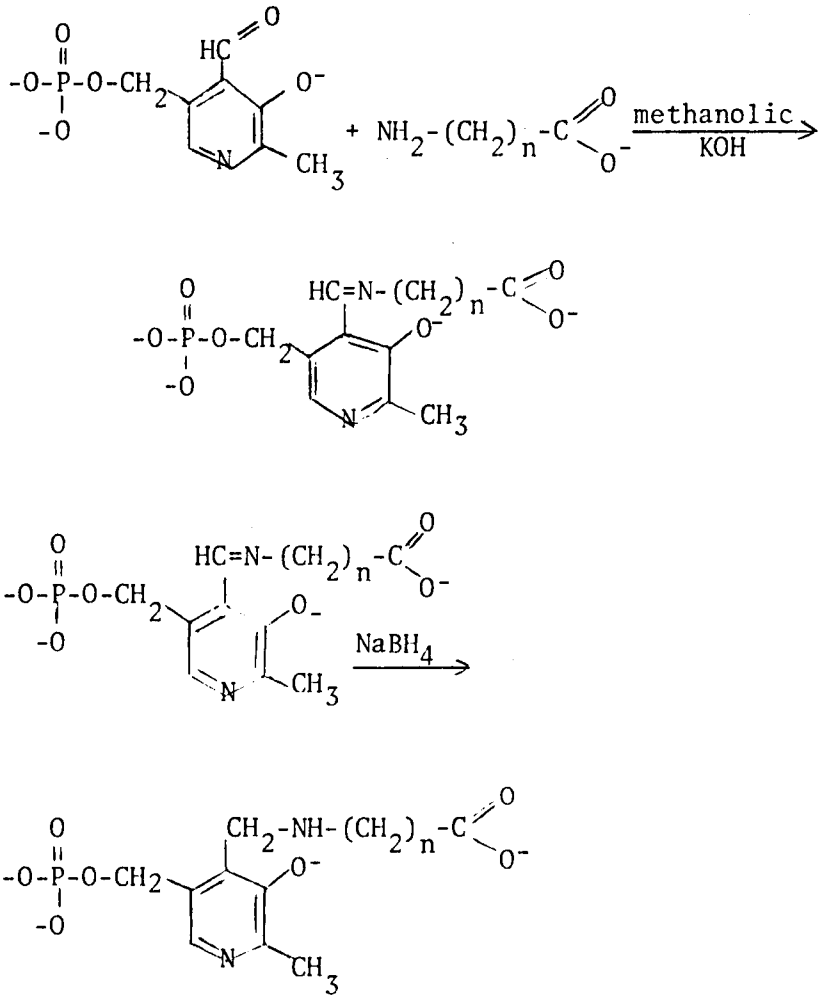
The conclusions drawn from the experiments with PNP oxidase are validated by the above examples. Multiple forms of the oxidase can have the same molecular weight and yet differ in their charge to mass ratio. This difference can arise from several types of alterations, the most obvious being a difference in the primary structure of the enzyme. A more definitive characterization of the oxidase forms cannot be achieved without each being purified to homogeneity.

CHAPTER IV

PHOSPHOPYRIDOXYL ANALOGUE STUDIES

Materials and Methods

Analogue synthesis—the reaction is represented below:



Twenty-five milligrams of PLP was dissolved in 30 ml of methanolic potassium hydroxide (MeKOH). To this solution was added the appropriate

amino carboxylic acid in a five-fold excess with respect to PLP. The amino carboxylic acids used were β-alanine (n = 2), γ-aminobutyric acid (n = 3), δ-aminovaleric acid (n = 4), and ω-aminocaprylic acid (n = 7). The solution was refluxed for 2 hours. The Schiff's base was reduced with sodium borohydride ($NaBH_4$). Complete reduction was indicated by the disappearance of the intense greenish-yellow color. Upon complete reduction, the methanol was evaporated. The sample was dissolved in water and acidified with formic acid to pH 7.

The sample was applied to a 1 × 15 cm Dowex 1-X8 anion exchange chromatography column. The counter ion was a formate group. Elution was achieved using a 0.01 M formic acid pH step gradient with decreasing pH increments of 0.5.

Criteria for purity were based on two different procedures. Paper chromatography with n-butanol, acetic acid, and water (17:5:8) as the solvent system was used to detect unreacted aminocarboxylic acids. The unreacted aminocarboxylic acids were visualized with ninhydrin. The absorption spectra of PNP has a characteristic absorption maximum at 323 nm at pH 7. In borate buffer of the same pH, the peak shifts to 293 nm as a result of the borate complexing with the 3-OH and the 4-α-OH. The analogues did not exhibit any shift in absorbance maxima in the presence of the borate buffer, thus showing no contamination of PNP.

Phosphopyridoxyl-m-aminobenzoic acid was donated by Mr. Kim Doo Sik.

Enzymatic assays were previously described in Section III. Instrumentation was previously described in Section III.

Results and Discussion

Previous characterization of PNP oxidase from liver has resulted in the following conclusions (11). The K_M and V_{max} for PNP and pyridoxamine-5-phosphate (PMP) are similar. These conclusions were drawn from results obtained by time-lapse assays. Using a direct assay capable of monitoring the reaction time course, the reaction rates for PNP and PMP under saturating conditions are dissimilar. Also, the concentration of PMP required for saturation was found to be 10-fold excess over PNP.

Enzymatic activity as a result of the utilization of either of these substrates was examined as a function of pH. These results are shown in Figure IV.1. Enzymatic activity (nm/min) is graphed against pH. As the pH increases, so does the enzymatic activity. The rate of the reaction observed for PNP is always higher than that observed for PMP.

The increase in the reaction rate as a function of pH is more dramatic for PMP than observed for PNP. These results suggest that as the pH increases, the primary amino group becomes unprotonated, thereby facilitating a nucleophilic attack on the oxidase.

In the literature, secondary phosphopyridoxyl amines have been reported as PNP oxidase substrates, such as phosphopyridoxyl-β-alanine (11, 12). A series of similar analogues were synthesized in which the methylene chain separating the amino group from the carboxyl group was extended. Each compound was incubated with PNP oxidase at a saturating concentration. The rate of PLP formation was obtained spectrophotometrically and the rates were compared to that obtained for PNP. The data are summarized in Table II.

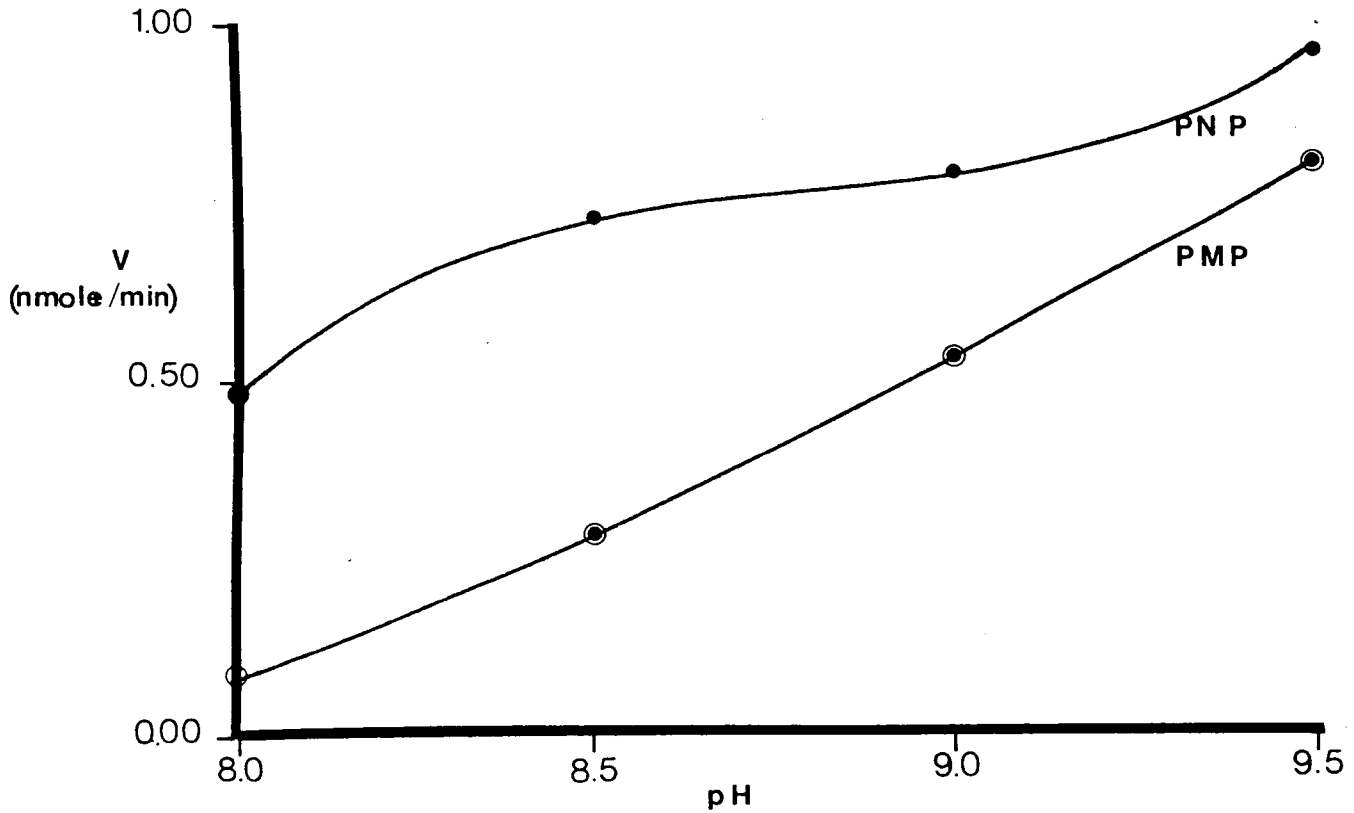


Figure IV.1. Relation of pH to rate of oxidation for PNP (●) and PMP (◎) by PNP oxidase. For pH 8.0 and 8.5, 0.1 M potassium phosphate was used. For pH 9.0 and 9.5, 0.1 M sodium pyrrophosphate was used.

TABLE II

SUMMARY OF RESULTS FOR PHOSPHOPYRIDOXYL ANALOGUES

Compound[a]	n[b]	V_{max} (nm/min)	V_{max}/V'_{max}[c]
PNP	-	0.47 ± 8%	1.00
pp-β-alanine	2	0.22 ± 4%	0.47
pp-γ-aminobutyric acid	3	0.23 ± 4%	0.49
pp-δ-aminovaleric acid	4	0.24 ± 8%	0.51
pp-ω-aminocaprylic acid	7	0.10 ± 6%	0.21
PMP	-	0.08 ± 6%	0.17

[a] pp is the abbreviation for phosphopyridoxyl.

[b] n = the number of methylene groups separating the amino group from the carboxyl group.

[c] V'max is the reaction rate observed for PNP.

Phosphopyridoxyl-β-alanine, phosphopyridoxyl-γ-aminobutyric acid, and phosphopyridoxyl-δ-aminovaleric acid exhibit the same rate of product formation. Phosphopyridoxyl-ω-aminocaprylic acid yields a 40% reduction in the rate of product formation when compared to the rate obtained for the other analogues. The reaction rates of the analogues are intermediate compared to those obtained for PNP and PMP.

Increase in the methylene carbon chain, from two to four carbons, yields no effect on the reaction rate, whereas an increase from four to seven carbons results in a decrease in rate. These data imply that the active site is large enough to accommodate aliphatic carboxylic acids with a chain length up to four methylene groups. Further extension of the chain to seven methylene groups implies impairment of substrate accommodation by the active site.

Further analysis of active site properties was investigated using phosphopyridoxyl-m-aminobenzoic acid (pp-m-aminobenzoic acid). The substituent group, m-aminobenzoic acid, is aromatic and it is more bulky than the aliphatic carboxylic acids. The K_M and k_{cat} were obtained for this compound along with PNP, PMP, and phosphopyridoxyl-γ-aminobutyric acid. These data are summarized in Table III.

Using the Michaelis-Menten Equation:

$$v = \frac{V_{max}\ (S)}{K_M + (S)} \qquad \text{(IV.1)}$$

For $(S) \ll K_M$ and $(E_o) \approx (E)$

TABLE III

SUMMARIZATION OF THE KINETIC PARAMETERS OBTAINED FOR PHOSPHOPYRIDOXYL ANALOGUES

Compound	$k_{cat}(sec^{-1})$	$K_{M}(uM)$	$k_{cat}/K_{M}(sec^{-1}M^{-1})$
PNP	0.021	13	1.62×10^{3}
PMP	0.008	100	0.08×10^{3}
pp-γ-aminobutyric acid	0.012	28	0.43×10^{3}
pp-m-aminobenzoic acid	0.026	1[a]	26.00×10^{3}

k_{cat} and K_{M} were determined using 0.1 M sodium pyrrophosphate buffer, pH 8.4.

[a]Due to equipment limitations, only an upper limit for the K_{M} value could be determined.

The velocity, v, is given by equation

$$v = \frac{k_{cat}}{K_M} (E) (S) \qquad (IV.2)$$

The term k_{cat}/K_M is a bimolecular rate constant which directly relates the concentration of enzyme and substrate to the velocity of the reaction.

It may be seen that an increase in k_{cat} and a decrease in K_M results in a dramatic increase in the rate of the reaction. The reaction rate for pp-∂-aminobutyric acid is 5-fold greater than that obtained for PMP. Replacement of the primary amine with m-aminobenzoic acid results in a 60-fold increase in the reaction rate compared to pp-γ-aminobutyric acid. Thus, attachment of the aromatic structure stabilizes the transition state, i.e., increases the k_{cat}; and facilitates binding of the substrate, i.e., decreases the K_M.

In the initial part of this section, a pH effect was observed when PMP was used as a substrate. The large increase in activity as the pH was increased from 8.0 to 9.5 was indicative of the deprotonation of the primary amine (pK 10). It should be noted that the pK's for aliphatic and secondary amines are around 10, with the secondary amine being slightly lower (0.3 to 0.4 pK units lower than the primary amines) (9). Furthermore, the replacement of the aliphatic group with an aromatic group decreases the pK to 5, thereby changing the amine from a strong base to a weak acid.

If the mechanism of PNP oxidase involves the formation of an enamine or an imine intermediate:

$$R = \underset{\text{enamine}}{CH - NH - R'} \longleftrightarrow R - \underset{\text{imine}}{CH = \overset{+}{N}H - R'}$$

then a decrease in the pK value of the secondary amine would facilitate the formation of such an intermediate. Also, the aromatic ring offers resonance energy stabilization to this type of intermediate, once it is formed.

A plausible reaction mechanism for the conversion of an amine to an aldehyde involves two main steps. First, there is the removal of a hydride which results in the enamine formation. Loss of a proton results in the interconversion of the enamine to an imine. Hydride, proton oxidation is common to flavin-catalyzed reactions (21). Protonation of the imino nitrogen followed by addition of a hydroxyl group to the electrophylic carbon results in the aldehyde formation and the amino group is liberated.

In summary, the analogue experiments offer the final conclusions:

1. PNP is the preferred substrate in reference to PMP. This holds true for a pH range of 7.0 to 9.5.

2. The active site dimensions in contact with the 4-α-carbon of the pyridine ring is large and has the capability of accommodating substituent groups from 5 Å to 7 Å in length (distances were estimated from the fully extended form of the chains using framework molecular models). This distance is coincident with the length of the flavin, indicating a possible interaction between these two moieties.

3. Replacement of an aliphatic carboxylic acid with an aromatic carboxylic acid greatly reduces the K_M and increases the k_{cat}. This

effect can be attributed to a decrease in the amino group pK, thus facilitating transition state formation. Stabilization is increased by the additional resonance energy. The aromatic group can also increase the affinity of the enzyme for the initial form of this compound. The increased affinity can be attributed to the π-π interaction between the benzoic acid and aromatic amino acid R groups of the enzyme or the conjugated ring system of the flavin, or a combination of the two.

CHAPTER V

DETERMINATION OF SEPARATION DISTANCE BETWEEN FMN AND SUBSTRATE BINDING SITE OF PNP OXIDASE

Materials and Methods

For an apo-oxidase preparation, holo-oxidase was obtained using the purification scheme described in Section III. Apo-oxidase was prepared using the method of Mayhew (14). The oxidase was dialyzed against 500 volumes of 2M KBr in 0.1 M potassium acetate buffer, pH 4.0, for 48 hours, after which time the pH was readjusted to pH 6.8 by dialysis with 0.01 M potassium phosphate buffer, pH 6.8.

Quantumn Yield Determination of Phosphopyridoxyl Oxime

The quantumn yield was determined in reference to quinine bisulfate. The absorbance of quinine bisulfate is equal to the absorbance of PLP-oxime at 350 nm. Quinine bisulfate and PLP-oxime were excited separately at 350 nm and the fluorescence emission was recorded. The area under each spectra was obtained. Using a quantumn yield of 0.5 for the quinine bisulfate, a value of 0.09 was obtained for the quantumn yield of the oxime.

Instrumentation

Absorption spectra were obtained with a Cary 15 spectrophotometer. Fluorescence emission spectra were obtained using the spectrofluorimeter described in Section I. Polarization of fluorescence was performed using

an instrument equipped with a 150 watt xenon lamp excitation light source. The excitation wavelength was selected by a quartz prism monochromator. Fluorescence emission wavelength was selected by using a Corning filter with a cutoff at 400 nm. The signal was monitored using an amplifier coupled with a digital voltmeter.

The compound phosphopyridoxyl-oxime, and its fluorescence lifetime, were obtained by Ms. Christine Wu.

Results and Discussion

PNP oxidase contains a single bound flavin per enzyme molecule. The role of the flavin in catalysis is of an oxidation-reduction nature. The oxidation of the substrate by the flavin is a proposed model for catalysis. There is no direct evidence showing the existence of a reduced or semi-reduced flavin during the process of catalysis. Indirect or direct attack on the substrate by the flavin can be evaluated by determining the separation distance between the flavin and the substrate binding site.

Determination of the proximity existing between the flavin and the substrate binding site was accomplished by resonance transfer experiments. A fluorescent inhibitor, phosphopyridoxyl-oxime, was used as a donor and the enzyme-bound flavin was used as the acceptor.

The critical distance of transfer was determined using Förster's equation (V.1):

$$R_o = \left[\frac{9 \times 10^{-25} \cdot Qo \cdot X^2 \cdot J}{n^4}\right]^{1/6} \qquad (V.1)$$

where Q_o, X^2, n, J, represent the quantumn yield of the donor in the absence of acceptor, the orientation factor, refractive index, and overlap integral. The overlap integral is obtained using equation V.2,

$$J = \frac{\Sigma\varepsilon(\lambda)\ F(\lambda)\ \lambda^4}{\Sigma F(\lambda)} \qquad (V.2)$$

where $\varepsilon(\lambda)$ represents the extinction coefficient of the acceptor at each wavelength and $F(\lambda)$ represents the fluorescence emission of the donor at each wavelength. The data from Figure V.1 yield $J = 2.245 \times 10^{-14} cm^6/$ mmole. Using this value along with an orientation factor of 2/3 and a refractive index of 1.33, $R_o = 25$ Å.

The actual distance of transfer is determined by equation V.3,

$$E = 1 - F/F_o = \frac{1}{1 + (R/R_o)} \qquad (V.3)$$

where E represents the efficiency of energy transfer, F and F_o are the fluorescence emissions of the donor in the presence and absence of acceptor, and R is the actual distance of transfer.

The fluorescence emission spectra of the free oxime, and oxime in the presence of holo and apo-oxidase are illustrated in Figure V.2. The F and F_o values obtained yielded R = 17 Å.

Quenching of fluorescence emission was carried out using 3.45×10^{-6}M oxime in the presence of 1.125×10^{-5}M oxidase. To ensure that all the ligand is bound to the enzyme, a polarization of fluorescence was determined at room temperature. A value of 0.20 was obtained for the oxime in the presence of oxidase and a value of 0.04 was obtained in the

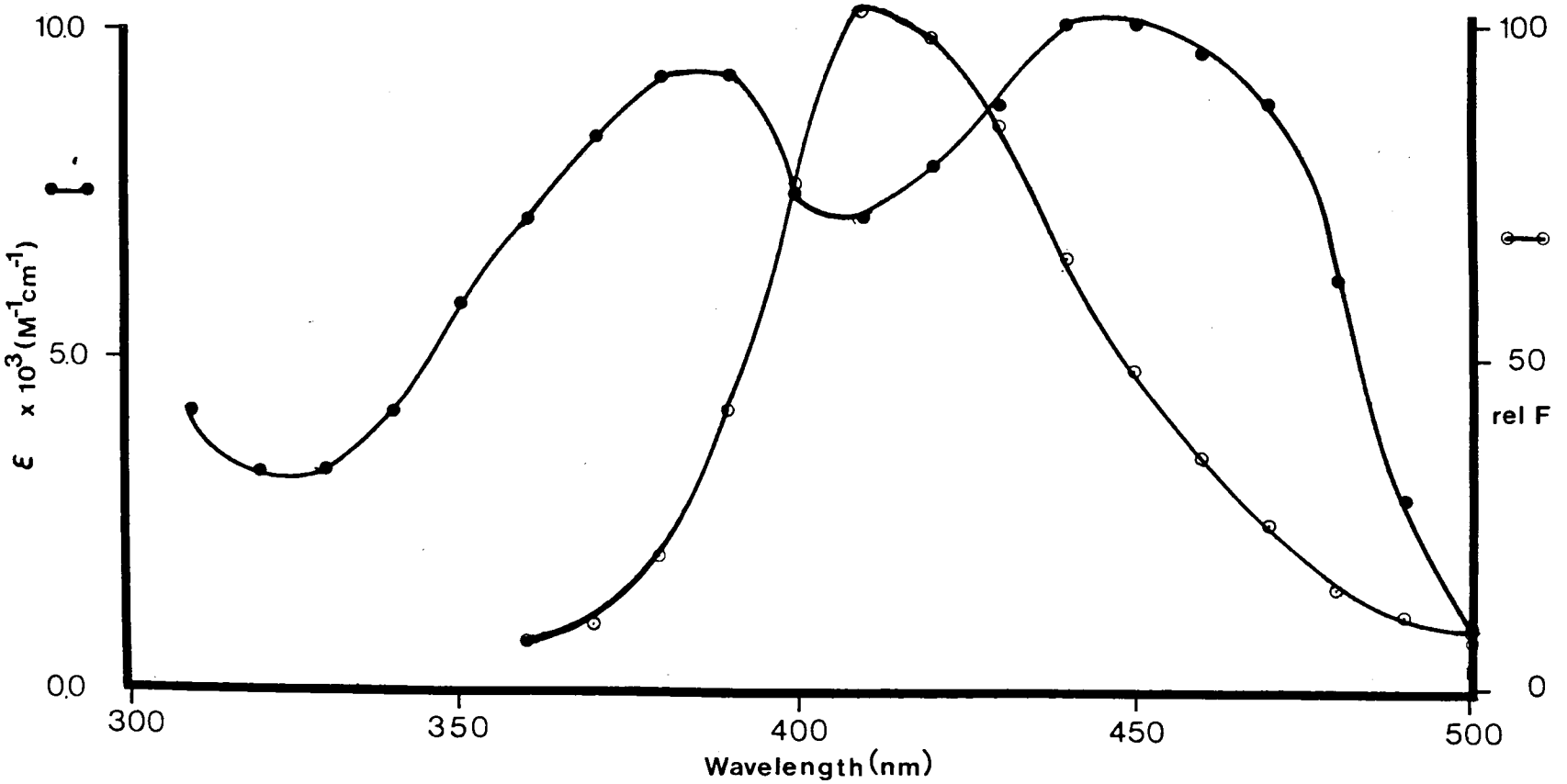


Figure V.1. Fluorescence emission spectra of PLP-oxime and the absorption spectra of FMN bound to PNP oxidase. Spectra were obtained in 0.05 M potassium phosphate buffer, pH 6.8. A spectral overlap integral, J, was obtained. $J = 2.45 \times 10^{-14} cm^6/mmole$. Oxime was excited at 330 nm.

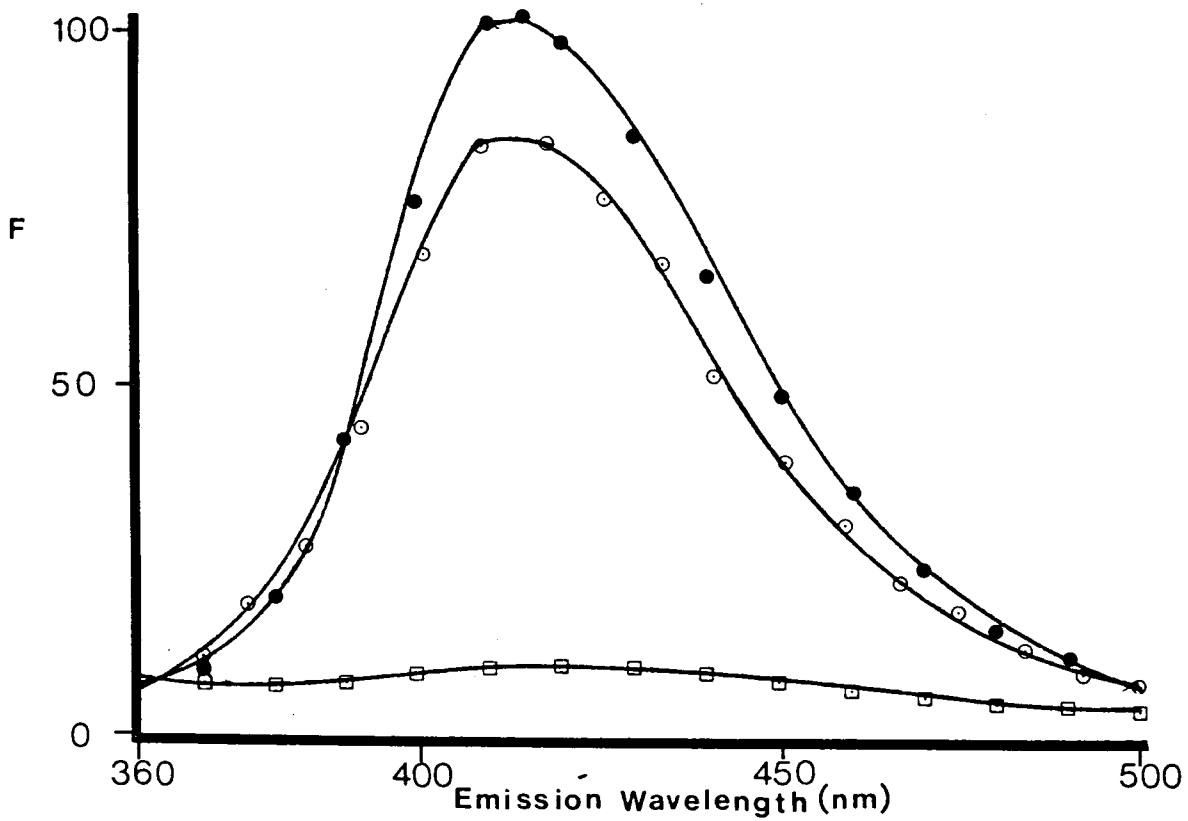


Figure V.2. Emission spectra of PLP-oxime (3.4 uM) (●), PLP-oxime (3.4 uM) + Apo-PNP oxidase (10 uM) (o), and PLP-oxime (3.4 uM) + Holo-PNP oxidase (10 uM) (⊡) excited at 330 nm. Spectra were obtained in 0.05 M potassium phosphate buffer, pH 6.8.

absence of oxidase. In comparison, PLP-oxime bound to bovine serum albumin yielded a polarization value of 0.26.

The degree of polarization increased dramatically for the oxime in the presence of holo-oxidase, and the value is comparable to that obtained for oxime bound to bovine serum albumin.

The efficiency of transfer was obtained on the basis of oxime fluorescence quenching. This type of quenching may involve several types of interactions besides resonance energy transfer. These other types of quenching are dependent upon collisional processes and are therefore diffusion controlled.

The rate of fluorescence quenching can be obtained using the Stern-Volmer relation:

$$\frac{\phi_f^o}{\phi_f} = 1 + \frac{k_Q\,(Q)}{k_f + k_t + k_p} \qquad (V.4)$$

where ϕ_f^o and ϕ_f are equal to the quantumn yield in the absence and presence of quencher, respectively. The terms k_Q, k_f, k_t, and k_p represent the rate constants for quenching, fluorescence, thermal deactivation, and photochemistry, respectively.

The fluorescence lifetime of the donor in the absence of quencher is represented by equation V.5:

$$\tau = \frac{1}{k_f + k_t + k_p} \qquad (V.5)$$

The quantumn yield ratio, ϕ_f^o/ϕ_f, can be represented by F^o/F. Substituting this relation along with equation V.5 into equation V.4 yields equation V.6,

$$F^o/F - 1 = k_Q \tau (Q) \qquad (V.6)$$

Using a lifetime value of 4.32 nsec and a quenching concentration of 1.125×10^{-5}M, $k_Q = 2.7 \times 10^{14} M^{-1} sec^{-1}$.

For a diffusion-controlled reaction, the reaction rate (rate of encounters/unit time) can be determined by equation V.7,

$$k_D = \frac{4}{1000} (r_M + r_N) (D_M + D_N) N_o \qquad (V.7)$$

where r and D represent the radii and diffusion coefficient for each of the reacting species, and k_D is the bimolecular rate constant. The diffusion coefficients for phosphopyridoxyl-oxime and PNP oxidase were estimated to be $7 \times 10^{-6} cm^2/sec$ and $7 \times 10^{-7} cm^2/sec$ (7, 8). The radii for the oxime and the oxidase were estimated to be 4 Å and 25 Å, respectively. Using these values, $k_D = 1.69 \times 10^{10} M^{-1} sec^{-1}$. This rate constant is based on the assumption that every encounter leads to product formation and therefore represents the upper limit.

This approximated rate constant for a diffusion-controlled reaction is much slower than the rate of quenching obtained previously. Therefore, the decrease in oxime fluorescence is attributed to some other form of energy transfer and not the result of collisional interactions.

The separation distance obtained is 17 Å. This distance is too large to result in a direct interaction between the substrate and the flavin.

For several types of flavins, a charge transfer complex has been observed to exist between the flavin and some form of the substrate (13). A maximum distance of separation for this type of interaction is 10 Å. To obtain this value using the overlap integral for the oxime and the flavin, an efficiency of transfer equal to 99.99% would have to be obtained. This value is not observed with the instrumentation used for these experiments.

However, one has to be cautious in the interpretation of these results. In fact, the possibility that Resonance Energy Transfer (Foster's mechanism) is no longer operative over distances of the order of 10 Å must be seriously considered. It is well established that at shorter distances the exchange mechanism becomes operative. Unfortunately, it is virtually impossible to calculate the efficiency of quenching due to the exchange mechanism using the spectroscopic properties of the donor and the acceptor (2).

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APPENDIXES

APPENDIX A

OXYGEN ELECTRODE ASSAY FOR OXYGEN-CONSUMING ENZYMES

Calibration—

Stock Solutions:

1% N-methylphenazonium methylsulfate (PMS)

1 mM NADH

4 mg/ml catalase

All stock solutions are prepared fresh and are prepared using the enzyme assay buffer.

Calibration Solution:

5.5 ml enzyme assay buffer

0.5 ml of PMS stock solution

0.5 ml of catalase stock solution

To the oxygen electrode chamber was added 1.5 ml of calibration solution. The chimney is placed on top of the chamber and the solution is allowed to equilibrate to the temperature of the water bath. Agitation is achieved by the use of a small magnetic stir bar and a magnetic stirrer. Two violent or too slow an agitation speed will affect the results. The desired sensitivity is chosen and the pen is set to 100% using the zero offset. The chimney is removed and a small amount of sodium dithionite is added to the chamber. The chimney is replaced and the pen is allowed to stabilize. The dithionite step is repeated to ensure that a true zero has been recorded. The deoxygenated calibration solution is removed by aspiration and the chamber is flushed several times with the assay buffer.

A fresh solution of the calibration solution is added to the chamber, agitation is begun and the chimney is replaced. The calibration solution is allowed to equilibrate for 5 minutes. Once equilibrated, 0.1 to 0.3 nmole of NADH is added to the calibration mixture through the chimney. The volume should not exceed 0.020 ml. The deflection is recorded and the NADH addition is repeated two additional times. The amount of oxygen consumed per addition of NADH is determined by the following equation:

$$c = \frac{100}{2X} \cdot \frac{n}{v}$$

where X, n, and v represent the amount of deflection, umoles of NADH per addition, and the reaction volume, respectively.

Enzyme Assay:

The amount of enzyme needed per assay is determined and this is prepared in a 5 to 10 ml stock with assay buffer. To the chamber is added 1.5 ml of enzyme mixture and allowed to equilibrate. During this equilibration period, the rate of oxygen consumption by the electrode is recorded and used as a background. Once the electrode oxygen consumption trace was recorded, the substrate is added in the same manner the NADH was added for calibration. The volume should not exceed 0.02 $\overset{o}{m}$l. Record the rate of consumption for at least 3 minutes and subtract from this the rate obtained in the absence of substrate. This yields the rate of catalysis.

Instrumentation:

The oxygen electrode chamber is water jacketed and the temperature is thermostatically regulated. The head of the electrode is covered by a permeable membrane which is held in place by a rubber gasket. The electrode is filled with a 4 M potassium chloride solution and is attached to a battery operated potentiometer which yields a signal of 0.6 volts. The potentiometer is attached to a recorder.

Materials:

NADH (β, type III), PMS, catalase (yeast extract), were purchased from Sigma.

Comments:

This type of enzymatic assay is useful in determining reaction rates of crude homogenates. This especially holds true for homogenates in which the turbidity is high and the use of spectrophotometric and spectrofluorometric methods are susceptible to light scattering. This methodology has been used to determine the specific activity of PNP oxidase in brain and liver homogenates. The assay can also be used to determine kinetic mechanisms where oxygen is the second substrate of a bisubstrate reaction.

Source: J. Robinson and J. M. Cooper (1970), Anal. Biochem. 33, pp. 390-399.

APPENDIX B

LIST OF ABBREVIATIONS

PNP = Pyridoxine-5-Phosphate

PMP = Pyridoxamine-5-Phosphate

PLP = Pyridoxal-5-Phosphate

AOA = Amino-oxy Acetic Acid

PLP-AOA = Phosphopyridoxyl-Amino-Oxy Acetic Acid

PLP-Oxime = Phosphopyridoxyl-Oxime

K_I = Dissociation Constant for Dissociation of Competitive Inhibitor-Enzyme Complex

SDS = Sodium Dodecyl Sulfate

FMN = Flavin Mono Nucleotide

VITA

Sean M. Sullivan was born in Elizabeth, New Jersey, on February 19, 1953. He graduated from Roselle Catholic High School in June of 1970. The following September, he entered Maryville College in Maryville, Tennessee, and in June 1974 received a Bachelor of Arts degree in chemistry.

In August of 1974, he was employed by Hoffman-La Roche to work in their Quality Control Division in Nutley, New Jersey, as a bench chemist. In June of 1975, he was transferred to the Roche Diagnostics Division in Sommerville, New Jersey. There, he was commissioned to work with their radioimmunoassay systems.

In September of 1977, Mr. Sullivan enrolled at The University of Tennessee, Knoxville, as a post baccalaureate student. In June of 1978, he was accepted to the Master's degree program in the Biochemistry Department, and in March 1981 he received the degree of Master of Science.