Brain Pyridoxal Kinase

PURIFICATION, SUBSTRATE SPECIFICITIES, AND SENSITIZED PHOTODESTRUCTION OF AN ESSENTIAL HISTIDINE*

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Pyridoxal kinase has been purified 2,000-fold from pig brain. The enzyme preparation migrates as a single protein and activity band on analytical gel electrophoresis. Pyridoxal kinase, 60,000 molecular weight, catalyzes the phosphorylation of pyridoxal (Km = 2.5 x 10⁻⁹ M) and pyridoxine (Km = 1.7 x 10⁻⁵ M). Pyridoxamine is not a substrate of the purified kinase. Irradiation of the kinase in the presence of riboflavin leads to irreversible loss of catalytic activity. Riboflavin binds to the kinase with a Kp = 5 μM 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 mol of enzyme. The amino acid residues 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.

The formation of pyridoxal-5-P from ATP, pyridoxal, and a divalent cation (Zn²⁺) is catalyzed by pyridoxal kinase, an enzyme which has been detected in various rat and bovine tissues (1). Procedures have been developed for the purification of the enzyme from bovine brain (2). More recently, it has been shown that the mechanisms by which brain slices concentrate extracellular vitamin B₆ depend on pyridoxal kinase (3).

Despite these studies which indicate the important role played by pyridoxal kinase in the metabolism and transport of vitamin B₆, little is known about the mechanism of action of this enzyme. No information is available about the amino acid residues responsible for binding the substrates and the amino acid residues which participate in the catalytic step. In our laboratory we have isolated and characterized pyridoxal kinase from pig brain (4). The aims of the present investigation are 2-fold. Firstly, to study the specificity of the reaction catalyzed by purified pyridoxal kinase and secondly, to investigate the amino acid residues critically connected with enzyme activity.

**EXPERIMENTAL PROCEDURES**

**Pyridoxal Kinase Preparation**—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 1 h. Enzyme preparation procedures were carried out generally at 4°C. A Waring Blender was used to prepare a 25% (w/v) homogenate in a solution of 0.075 M potassium phosphate (pH 6.8). The homogenate was centrifuged at 10,000 × g for 30 min, and the precipitate was discarded. The supernatant was then treated with (NH₄)₂SO₄. The precipitate obtained at 40 to 60% saturation was dissolved in 0.01 M potassium phosphate (pH 6.8), containing glutathione (0.1 mM), pyridoxal (0.05 mM), and ZnCl₂ (0.05 mM) (Buffer 1). It was then dialyzed against Buffer 1 and applied to a column (35 × 2.6 cm) of CM-Sephadex C-50 equilibrated with the same buffer. The column was eluted with Buffer 1, the enzymes pyridoxal kinase and pyridoxine-5-P oxidase are not retained by CM-Sephadex, whereas red contaminating proteins remain adsorbed to the supporting gel. The enzymatic active fractions were combined and applied to a column (25 × 2.6 cm) of hydroxypatite previously equilibrated with Buffer 1. The enzymes, pyridoxal kinase and pyridoxine-5-P oxidase, were eluted by using a linear gradient made with the equilibration buffer (250 ml) and the same volume of 0.3 M potassium phosphate (pH 6.8) containing pyridoxal (0.05 mM), pyridoxine-5-P (0.05 mM), and ZnCl₂ (0.05 mM) (Buffer 2). The enzyme pyridoxine-5-P oxidase is eluted at a concentration of potassium phosphate of approximately 0.1 M whereas pyridoxal kinase is eluted at a concentration of potassium phosphate of 0.15 M.

The fractions containing pyridoxine-5-P oxidase activity were combined, concentrated by ultrafiltration, and subjected to further purification. The fractions containing pyridoxal kinase activity were combined, dialyzed against Buffer 1, and applied to a column (100 × 2.6 cm) of Sephadex G-100 previously equilibrated with the same buffer. The enzyme was then chromatographed on a column (14 × 0.9 cm) of 4-deoxy-pyridoxyl-Sepharose equilibrated with Buffer 1. The enzyme is retained by derivatized Sepharose and eluted by increasing concentrations of potassium chloride. The elution of the protein fraction containing pyridoxal kinase activity occurs at a concentration of potassium chloride of 0.15 M. This last step in the purification of the kinase permits the separation of several contaminating proteins. The purified fraction of pyridoxal kinase migrates as a single protein and activity band on analytical gel electrophoresis. The results obtained with this purification procedure are included in Table I.

The fractions containing pyridoxine-5-P oxidase activity were dialyzed against 0.01 M potassium phosphate (pH 6.8) and applied to a column (15 × 1 cm) of Sephadex G-100 previously equilibrated with the same buffer. The enzyme was eluted by using a linear gradient made with the equilibration buffer and the same volume of 0.2 M potassium phosphate (pH 6.8). The fractions showing pyridoxine-5-P oxidase activity were sent through Sephadex G-100 equilibrated with 0.01 M potassium phosphate (pH 6.8). The enzyme was then applied to a P-pyridoxyl-Sepharose column (10 × 1 cm) and eluted in a stepwise fashion by different concentrations of potassium phosphate 0.1, 0.15, and 0.2 M. The enzyme was concentrated by ultrafiltration and stored at -12°C. Under these conditions, pyridoxine-5-P oxidase remains stable for at least 2 months.

The purified preparation obtained after affinity chromatography has a specific activity of 100 units/mg and a Kₘ for pyridoxine-5-P of 10 μM at pH 8. This specific activity is comparable in magnitude to that obtained with rabbit liver pyridoxine-5-P oxidase (5). A unit of specific activity is defined as the amount of protein which catalyzes the formation of 1 nmol of pyridoxal-5-P per min at 37°C. The purification procedure together with the properties of the enzyme will be published elsewhere.

**Enzymatic Assay**—For precise kinetic data, the formation of pyridoxal-5-P was measured by following the change in absorbance at 388 nm at which pyridoxal-5-P has an absorption maximum with an extinction coefficient of 4900 M⁻¹ cm⁻¹ at pH 7. Changes in absorbance

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Brain Pyridoxal Kinase

**Table I**

<table>
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<tr>
<th>Treatment</th>
<th>Volume (ml)</th>
<th>Protein (mg)</th>
<th>Protein concentration (mg/ml)</th>
<th>Specific activitya</th>
<th>Total activity units</th>
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<td>117,600</td>
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<td>8</td>
<td>6.4</td>
<td>0.8</td>
<td>7.13</td>
<td>456</td>
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</table>

*a A unit of activity is defined as that amount of protein which catalyzes the formation of 1 nmol of pyridoxal 5-phosphate per min at 37°C.

Pyridoxal kinase activity was detected by slicing an unstained gel into 1 mm sections, macerating the slices in 0.5 ml of 0.075 M potassium phosphate (pH 7) containing pyridoxal (0.1 mM), ATP (0.1 mM), and ZnCl2 (0.05 mM), allowing the suspension to stand overnight and assay each sample by the phenylhydrazine method (1).

Pyridoxine-5-P oxidase activity was detected by the method of Feinstein and Lindahl (9).

Gel filtration was used for the determination of the molecular weight of the purified enzymes. The degree to which a column (100 x 2 cm) of Sephadex G-150 separated macromolecules differing in molecular size was determined by preliminary experiments with proteins of known molecular weight. Samples of ribonuclease, chymotrypsin, bovine serum albumin, horse alcohol dehydrogenase, lactate dehydrogenase, and glutamate dehydrogenase were used as markers of known molecular weight. The elution volume of pyridoxal kinase corresponded to a molecular weight of 90,000, whereas that of pyridoxine-5-P oxidase corresponded to a molecular weight of approximately 50,000.

**Photoinactivation Experiments**—Photoinactivation experiments were carried out in a temperature-controlled cell compartment. The sample of enzyme (2 ml) in a glass cuvette (1-cm light path) was illuminated from the side with a 150-watt mercury lamp. The wave-length of excitation was selected by a Corning glass filter (C-S-0-52) which transmits light at wavelengths longer than 346 nm. The energy of the light used was measured by using a thermopile, Yellow Springs Instrument Company Inc.). Spectroscopic measurements were conducted in the following instruments. Absorption spectra were done in a Cary model 15 and in an Amino double beam spectrophotometer (DW2), fluorescence in an instrument equipped with two grating monochromators (Bausch and Lomb), and emission anisotropy in a polarization photometer (11).

The amino acid composition of native and photoactivated pyridoxal kinase was determined using resins type PA-35 and UR-30 on a Beckman amino acid analyzer (model 120B). Acid hydrolysis of the proteins was carried out in HCl at 110°C in sealed evacuated tubes for 24 h. The amino acid leucine was used as reference in calculating the number of photooxidized residues in the samples of inactivated enzyme.

The number of reactive SH groups were determined by reaction with DTNB using the procedure of Ellman (12). Titration of SH groups of the denatured enzymes were conducted in 6 M guanidine-HCl at 25°C. The molar extinction coefficient of 13,600 at 412 nm for thionitrobenzoic acid produced was employed for all calculations.

The number of tryptophan residues was determined by the colorimetric method of Spies and Chambers (13) and by reaction with N-bromosuccinimide in the presence of 8 M urea as described by Spande and Witkop (14). Protein concentration was determined by the method of Lowry et al. (15).

**Preparation of Vesicles Containing FMN**—Phosphatidylcholine vesicles were prepared according to the method of Huang (16). Lyophilized phosphatidylcholine (100 mg) was suspended in 2 ml of 0.07 M KCl containing 1 mM FMN. The suspension was ultrasonically irradiated (20 KHz) under nitrogen at 10°C for 1 h and then centrifuged at 105,000 x g. The resulting supernatant was then subjected to molecular sieve chromatography on a Sepharose 4B column (15 x 2 cm) previously equilibrated with 0.07 M KCl at 4°C.

The fractions eluted from the column were monitored at 300 and 445 nm by means of absorption spectroscopy. Two major peaks (Fractions I and II) were eluted from the column. Fraction I, which is very homogeneous with respect to both size and shape, was used throughout these studies. These data are not shown.

**Materials**—Sephase derivatized with N-('ω-aminoheptyl)-4-deoxypyriddylic acid was prepared by reacting the "spacer group" (N-('ω-aminoheptyl) of Sephase AH (Pharmacal) with 4-deoxy-5-pyridoxaldehyde (2,4-dimethyl-5-formyl-3 hydroxyprydine) at pH 7. Sephase AH (6 g) was suspended in 50 ml of water, brought to pH 7 by addition of NaOH, mixed with 1 g of 4-deoxy-5-pyridoxaldehyde, and allowed to react at 25°C in the dark for 12 h. The resulting Schiff’s base was reduced by the addition of 20 mg of solid sodium borohydride at 4°C. After reduction the derivatized Sephase was washed several times with water. 4-Deoxy-5-pyridoxaldehyde was prepared according to the method of Sattan and Argoudelis (17) by the oxidation of 4-deoxy-pyridoxine with manganese dioxide. Sephase derivatized with N-('ω-aminoheptyl)p-aminobenzenetiazide (PAP) was prepared according to the method of Lee and Churchich (18). The reagents, 4-deoxypyriddylic acid, pyridoxal, ATP, ADP, alpha-ATP, pyridoxal-5-P, 4-aminobenzenetiazide, and the danaylated derivatives of phenylalanine, tyrosine, and tryptophan.
RESULTS

Substrates of Pyridoxal Kinase—Although it is generally accepted that pyridoxal kinase phosphorylates all the non-phosphorylated vitamins, i.e. pyridoxal, pyridoxine, and pyridoxamine, it was thought worthwhile to investigate whether similar reactions take place in the presence of purified pyridoxal kinase.

The results obtained with all the substrates tested are included in Table II. The phosphorylation of pyridoxal, which is easily monitored by means of absorption spectroscopy, is characterized by a $K_m$ of $2.5 \times 10^{-5} \text{ M}$ at the pH optimum of 6. The catalytic activity of the kinase toward the substrate pyridoxine was studied by using an enzymatic assay system consisting of two purified enzymes, pyridoxal kinase and pyridoxine-5-P oxidase. As may be seen from the results included in Table II, the $V_{max}$ obtained for pyridoxine is only one-half of the $V_{max}$ obtained for pyridoxal. Thus, the rate of phosphorylation catalyzed by the kinase depends upon the chemical structure of the substrate.

The conversion of pyridoxamine-5-P to pyridoxal-5-P is catalyzed by the oxidase (3), but we were unable to detect the formation of pyridoxal-5-P when ATP and pyridoxamine were used as substrates in the presence of both enzymes, i.e. pyridoxal kinase and pyridoxine-5-P oxidase. In addition, it was surprising to find that pyridoxamine at concentrations of 1 mM failed to exert any inhibitory effect on the phosphorylation of pyridoxal catalyzed by the kinase.

These unexpected results prompted us to investigate the ability of the kinase to phosphorylate the three vitamins, i.e. pyridoxal, pyridoxine, and pyridoxamine by using a method based on the reconstitution of aspartate aminotransferase activity. When the apoprotein of the transaminase, together with pyridoxal kinase and pyridoxine-5-P oxidase, was incubated at 25°C in the presence of ATP and pyridoxal, it was observed that the recovery of aminotransferase activity proceeded in the manner depicted in Fig. 1.

An initial lag in the reconstitution profile is followed by a linear recovery of aminotransferase activity as a function of the time of incubation. Maximum recovery of aminotransferase activity was detected after 40 min of incubation when the concentration of pyridoxal-5-P generated by the kinase was enough to saturate the binding sites of the apotransaminase.

With pyridoxine and ATP as substrates in the reaction mixture, the recovery of aminotransferase activity proceeded at a slower rate as shown by the results included in Fig. 1. Thus, essentially 20% of the aminotransferase activity is recovered within 40 min of incubation at 25°C. This observation is consistent with the finding that pyridoxal is a better substrate than pyridoxine in the phosphorylation step catalyzed by the kinase (Table II).

As expected, the addition of pyridoxamine and ATP to the reaction mixture consisting of three enzymes failed to elicit any recovery of aminotransferase activity even after prolonged times of incubation at 25°C.

Hence, the two methods used to study the specificity of the reaction catalyzed by the kinase clearly show that pyridoxamine is not phosphorylated by pyridoxal kinase.

Inhibitors of Pyridoxal Kinase—It has been shown previously (1) with partially purified preparations of enzyme that pyridoxaloximes are powerful inhibitors of pyridoxal kinase. This is also true of the purified preparation of pig brain which is inhibited by pyridoxaloxime with a $K_i$ of $2 \times 10^{-5} \text{ M}$. Several pyridoxal analogues, tested as inhibitors of the purified kinase, yielded the inhibition constants and inhibition patterns recorded in Table II. Pyridoxaloxime and 4-deoxypyridoxine inhibit pyridoxal kinase by competing with the substrate pyridoxal. Another significant result of these inhibition studies is the finding that among several danylated derivatives tested, Dns-4-aminobutyrate is a powerful reversible inhibitor of the kinase. The inhibitory effect exerted by Dns-4-aminobutyrate is not reversed by addition of potassium phosphate at concentrations greater than 70 mM, indicating that Dns-4-aminobutyrate does not compete with the monovalent potassium ions for the same binding site on the enzyme. Moreover, the initial velocity patterns obtained with ATP as the variable substrate and the danylated derivative as the changing, fixed inhibitor revealed that Dns-4-aminobutyrate is a competitive inhibitor with respect to ATP. Dns-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.

Binding of Riboflavin—Prior to the photoinactivation experiments, the interaction between pyridoxal kinase and riboflavin was studied by means of fluorescence spectroscopy. At a protein concentration of 10 $\mu$M and riboflavin concentration of 10 $\mu$M, 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 (10 $\mu$M) and riboflavin (2 $\mu$M) is considerably greater ($\Delta = 0.23$) 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, the fluorescence intensity and emission anisotropy \((A)\) of samples containing fixed concentrations of enzyme and varying concentrations of riboflavin were measured upon excitation with the light of 360 nm.

As shown in Fig. 2, the difference between the observed emission anisotropy \((\tilde{A})\) 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 \((a)\) is related to \(\tilde{A}, A_F, A_B, \) and \(\beta\), according to Equation 2

\[
a = \frac{\tilde{A} - A_F}{(A_B - A_F) \beta + (A - A_F)}
\]

It is possible to determine \(a\) from the titration curve given in Fig. 2 by resorting to Equation 3

\[
a = \frac{\tilde{A} - A_F}{A_B - A_F}
\]

which is applicable to the determination of \(a\) when the ratio of fluorescence yields for bound \((qB)\) and free ligand \((qF)\) is equal to one \((\beta = qB/qF)\). Since the binding of riboflavin to the enzyme does not elicit any change in the fluorescence yield, the use of Equation 3 is fully justified. When the titration results were analyzed by plotting \(\tilde{v}/[L]\) versus \(\tilde{v}\), it was found that the straight line intersects the abscissa at \(\tilde{v} = 0.9\) (Fig. 2, inset). Thus, there is one binding site on the protein surface to which riboflavin binds with an equal dissociation constant of 5 \(\mu M\).

The binding of riboflavin to the enzyme does not affect the catalytic function of pyridoxal kinase as demonstrated by the fact that the \(K_m\) and \(V_{max}\) values of the enzyme remain essentially unchanged in the presence of 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.

In view of the preceding results, we decided to investigate whether riboflavin acts as an efficient photosensitizer of amino acids implicated in catalytic reactions. For the photooxidation experiments, samples of the enzyme (10 \(\mu M\)) in 70 mM phosphate buffer (pH 6) were mixed with 1-, 2-, and 5-fold molar excess of riboflavin and irradiated with light of wavelengths longer than 340 nm at 10°C. Under this set of experimental conditions, the enzyme is soluble, no turbidity was observed during photolysis, and the samples are suitable for spectroscopic studies.

Fig. 3 shows the results obtained upon exposure to light of a mixture containing enzyme (10 \(\mu M\)) and riboflavin (10 \(\mu M\)), where it may be seen that in the presence of equimolar concentration of riboflavin, the enzyme undergoes a loss of enzymatic activity which follows first order kinetics. The addition of the substrate ATP (1 mM) to the mixture containing enzyme and riboflavin has a small effect on the rate of inactivation as shown by the results included in Fig. 3. However, protection against photoinactivation is enhanced when the competitive inhibitor Dns-4-aminobutyrate is added to the incubation mixture (Fig. 3).

During the photoinactivation experiments, a small decrease in the absorbance of the sensitizer (riboflavin) was detected, and the decrease in absorbance at 455 nm as a function of the time of irradiation follows the pattern included in Fig. 3. Similar changes in absorbances at 360 and 455 nm were recorded during irradiation of riboflavin in the absence of enzyme, indicating that the absorbance changes at either 360 or 455 nm are entirely due to photodestruction of the sensitizer.

** Destruction of Histidine Residues—Experiments conducted in several laboratories have shown that histidine and to a certain extent tyrosine and tryptophan are the amino acids more susceptible to chemical modification upon illumination in the presence of flavins (19).**

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 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 min (Table III). No significant change in cysteine, tyrosine, and tryptophan content was detected in extensively photooxidized samples. The finding
that the tryptophan content of the irradiated sample is, within experimental error, identical with the tryptophan content of native pyridoxal kinase, is consistent with the observation that the emission spectrum of the irradiated enzyme is indistinguishable from that of the native enzyme (Fig. 4).

Mechanism of Photosensitization—Several lines of experimental evidence indicate that singlet oxygen, generated by the transfer of excitation energy from the sensitizer to oxygen, is responsible for the photoxidation of histidine residues in proteins. It is highly possible that singlet oxygen is also involved in the photoactivation 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 2 µs in water solutions, is deactivated by strong quenchers such as β-carotene (19). The second experiment was to demonstrate that even when riboflavin is not bound to the enzyme, the photoactivation of pyridoxal kinase proceeds through the intermediacy of singlet oxygen.

As might be anticipated, the presence of β-carotene in the reaction mixture containing pyridoxal kinase and riboflavin should decrease the concentration of singlet oxygen available for reaction with the enzyme; consequently, the rate of photoactivation in the presence of β-carotene should be diminished.

This expectation is born out by the results included in Fig. 3, where it may be seen that β-carotene indeed decreases the rate of photoactivation.

In order to demonstrate that binding of riboflavin to the enzyme is not required for the inactivation of pyridoxal kinase, photoirradiation experiments were conducted in the presence of phosphatidylcholine vesicles carrying the photosensitizer FMN.

When samples irradiated for different lengths of time were assayed for enzymatic activity, it was found that the enzyme undergoes an irreversible loss of catalytic activity (Fig. 5). Samples of pyridoxal kinase mixed with phosphatidylcholine vesicles remain fully active provided they are not exposed to irradiation as indicated by the results included in Fig. 5.

### Table III

<table>
<thead>
<tr>
<th>Irradiation time</th>
<th>Activity</th>
<th>Sulfhydryl Residues</th>
<th>Tryptophan Residues</th>
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<tr>
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</table>

* Determined by the colorimetric method of Spies and Chambers (13) and by reaction with N-bromosuccinimide (14).

![Fig. 4. Emission spectra of native (●) and photoactivated (△) pyridoxal kinase in 70 mM potassium phosphate (pH 6). Excitation wavelength 290 nm. Both samples have the same absorbance (A = 0.1) at the exciting wavelength.](http://www.jbc.org/)

![Fig. 5. Loss of pyridoxal kinase activity sensitized by FMN. Enzyme (10 µM) irradiated in the presence of phosphatidylcholine vesicles containing FMN (30 µM) (○) and enzyme (10 µM) irradiated in the presence of FMN (30 µM) (●) at pH 6 in 70 mM potassium phosphate with an intensity of light of 9 × 10^5 ergs cm^-2 s^-1 sample of pyridoxal kinase (10 µM) mixed with phosphatidylcholine vesicles containing FMN (30 µM) and kept in the dark (●). Experiments conducted at 10°C.](http://www.jbc.org/)

Since the efficient photoactivation 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.

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 is the case, then rechromatography of irradiated vesicles should indicate changes in the elution profiles as well as variations in the concentrations of FMN. The results of these experiments are included in Fig. 6, where it may be seen that rechromatography of irradiated vesicles resulted in a single peak eluting at a volume corresponding to its position in the original chromatogram.

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

Reactivity of Cysteine and Tyrosine Residues—The preceding results indicate that the photodynamic action of riboflavin is related to its ability to sensitize the chemical modification of histidyl residues. However, it was thought of interest to demonstrate by independent methods that the amino acids cysteine and tyrosine are not involved in the binding of the substrates pyridoxal and A11'.

The kinetics of the reaction of the thiol groups of the enzyme with DTNB was studied in the presence of excess DTNB under pseudo-first order conditions at 25°C. Fig. 7 shows the results obtained when the reaction was monitored at 412 nm. 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 412 nm. 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 Fig. 7.
The increase in absorbance at 428 nm corresponds to the full catalytic activity (results not shown). Despite the nitration of 2 tyrosine residues, the enzyme retains reaction of approximately 2 mol of tyrosine per mol of enzyme. The reaction of tetranitromethane with the tyrosine residues of pyridoxal kinase was studied under experimental conditions that decrease the reactivity of thiol groups (20). In these conditions the reaction is complete within 30 min, and the enzyme retains full catalytic activity (results not shown).

**DISCUSSION**

The studies reported in this paper have shown that flavins are efficient photosensitizers of histidine residues critically connected with the catalytic function of pyridoxal kinase. The results of the amino acid analyses indicate that no change in the cysteine, tyrosine, and tryptophan content of 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 of pyridoxal kinase is influenced by the presence of the substrate ATP or by addition of the strong competitive inhibitor Dns-4-aminobutyrate. 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 (21). In the first class, Type I mechanism, the triplet state of the sensitizer interacts with another molecule directly, usually with hydrogen atom or electron transfer. The radical thus produced undergoes chemical reactions with other organic molecules, i.e., amino acids.

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 (22), 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 (23).

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 by quenchers of singlet oxygen such as β-carotene.

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

**REFERENCES**

Brain Pyridoxal Kinase

Brain pyridoxal kinase. Purification, substrate specificities, and sensitized photodestruction of an essential histidine.

F Kwok and J E Churchich


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