J. Biochem. Mol. Biol. Vol. 30, No. 2, pp. 125~131 March 31, 1997

Catalytic and Structural Properties of Pyridoxal Kinase

Jung Jong Cho, Se-Kwon Kim[1] and Young Tae Kim*

Department of Microbiology, [1]Department of Chemistry, Pukyong National University, Pusan 608-737, Korea
(Received January 10, 1997)

Abstract : This work reports studies of the catalytic and structural properties of pyridoxal kinase (ATP: pyridoxal 5′-phosphotransferase, EC. 2.7.1.35). Pyridoxal kinase catalyzes the phosphorylation of vitamin B_6 (pyridoxal, pyridoxamine, pyridoxine) using ATP-Zn as a phosphoryl donor. The enzyme purified from brain tissues is made up of two identical subunits of 40 kDa each. Native enzyme was inhibited by a substrate analogue, pyridoxal-oxime. Limited chymotrypsin digestion of pyridoxal kinase yields two fragments of 24 and 16 kDa with concomitant loss of catalytic activity. These fragments were isolated by DEAE ion exchange chromatography and used for binding studies with fluorescent ATP and pyridoxal analogues. The spectroscopic properties of both fluorescent pyridoxal analogue and Anthraniloyl ATP (Ant-ATP) bound to the 24 kDa fragment are indistinguishable from those of both pyridoxal analogue and Ant-ATP bound to the native pyridoxal kinase, respectively. The small 16 kDa fragment, generated by proteolytic cleavage of the kinase, does not bind any of the substrate analogues. Binding characteristics of Ant-ATP were extensively studied by measuring the changes in fluorescence spectra at various conditions. From the results presented herein, it is postulated that the structural domain associated with catalytic activity comprises approximately one-half of the molecular mass of pyridoxal kinase (24 kDa), whereas the remaining portion (16 kDa) of the enzyme contains a regulatory binding domain.

Key words : catalytic domain, limited proteolysis, pyridoxal kinase, pyridoxal-5′-P.

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 (Snell, 1990). The formation of pyridoxal-P catalyzed by pyridoxal kinase from pyridoxal, ATP and a divalent cation is essential for many transamination, decarboxylation and racemization reactions (McCormick *et al.*, 1961; Meister, 1990). Besides the conversion of pyridoxal to pyridoxal-P, this enzyme can also phosphorylate pyridoxine and pyridoxamine from ATP.

Procedures for the purification of pyridoxal kinase from bovine, porcine, and sheep brains have been developed in several laboratories (Neary and Diven, 1970; Kwok and Churchich, 1979; Kerry *et al.*, 1986). Recently, the enzyme isolated from sheep brain has been identified to be a dimer with a molecular weight of 80 kDa, and dissociation of the dimeric structure under mild denaturing conditions demonstrated that the monomeric species were catalytically competent (Kerry *et al.*, 1986). Similar to other well-characterized kinases (Bennet and Steitz, 1978; Weigard and Reminton, 1986), it seems likely that pyridoxal kinase contains a cleft that divides the monomeric protein into two lobes. Two structural domains connected by a flexible polypeptide chain might undergo conformational reorganizations prior to the catalytic events (Dominici *et al.*, 1988; Kim *et al.*, 1988; Kwok and Churchich, 1988; Churchich and Kim, 1990; Wolkers *et al.*, 1991).

Many pyridoxal analogs have been reported to be inhibitors of pyridoxal kinase (McCormick and Snell, 1961; Loo and Whittaker, 1967) and these analogs are primarily oximes, azines and hydrazines of pyridoxal. Biogenic amines have also been reported to inhibit pyridoxal kinase (Neary, 1972).

Previous physical studies have suggested that substrate binding sites of pyridoxal kinase are hydrophobic (Kwok, F and Churchich, 1979). In a recent report (Dominici *et al.*, 1988), the affinity label reagent, pyridoxal-ATP (AP_4-PL), has reacted with a specific lysyl residue of monomeric pyridoxal kinase. Another report (Scholtz and Kwok, 1989) showed that a tyrosine residue at the pyridoxal binding site is essential for catalytic activity. Furthermore, limited digestion of the enzyme with chymotrypsin resulted in a decrease in the catalytic activity, whereas the electrophoretic patterns revealed the presence of two bands of 24 kDa and 16

*To whom correspondence should be addressed.
Tel : 82-51-620-6366, Fax : 82-51-628-8147
E-mail : ytkim@dolphin.pknu.ac.kr

kDa, respectively, arising from the cleavage of the monomeric 40 kDa protein (Churchich and Kim, 1990). In an attempt to increase our understanding of the structure and function of pyridoxal kinase, we have decided to investigate the catalytic and structural properties of the enzyme. The ability of some fluorescent analogs of ATP and pyridoxal to bind to the enzyme can be exploited to gain further information on the structure and function of different structural domains.

Materials and Methods

Materials

Protein standards for electrophoresis experiments were obtained from Bio-Rad (Richmond, USA). Chymotrypsin and trypsin inhibitor were from Boeringer Mannheim (Indianapolise, USA). Sephadex LH-20 and DEAE-cellulose were purchased from Pharmacia (Uppsala, Sweden). Pyridoxal-5′-P, pyridoxal, ATP, and other chemicals were obtained from Sigma (St. Louis, USA).

Preparation of Anthraniloyl-ATP

The reagent anthraniloyl-ATP (Ant-ATP) was prepared essentially as described by Hirasuka (1983) for the ribonucleotides, except a threefold excess of isatoic anhydride over ATP was used. The product was purified by two chromatographic steps using a Sephadex LH-20 column (2 cm×50 cm) equilibrated with water and a DEAE-cellulose column (2 cm×30 cm) equilibrated with 0.2 M tetraethylammonium bicarbonate, pH 7.5, and eluted with a gradient of 0.2~0.5 M tetraethylammonium bicarbonate, pH 7.5.

The purified compound has absorption coefficients of 20,000 $M^{-1} \cdot cm^{-1}$ and 4,700 $M^{-1} \cdot cm^{-1}$ at 252 nm and 330 nm, respectively.

Purification of the kinase

Pyridoxal kinase from sheep brain was purified according to the procedure previously described by Kerry and Kwok (1986). The last step of the purification procedure includes a step of affinity chromatography on pyridoxal-Sepharose and elution with pyridoxal (10 mM) at pH 5.

Enzymatic assays

The rate of formation of pyridoxal-5′-P was measured by following the increase in absorbance at 388 nm, with an extinction coefficient of 4,900 $^{M-1} \cdot cm^{-1}$ at pH 7. Enzymatic activity was measured at pH 6.5 in 0.075 M potassium phosphate containing ATP (0.2 mM), pyridoxal (0.4 mM), and Zn^{2+}-acetate (0.2 mM) at 25°C. Initial rate measurements were carried out by monitoring the change in absorbance at 388 nm for at least 5 min in a double beam spectrophotometer (UVICON-922) at 0.5 absorbance full scale.

Limited proteolysis with chymotrypsin

Pyridoxal kinase (5 mg/ml) was incubated with chymotrypsin (0.01 mg/ml) in 0.1 M Na-phosphate (pH 7.0) at 25°C for 1 h. At the end of the incubation, 0.1 mg of trypsin inhibitor (soy bean) was added to the incubation mixture. Aliquots withdrawn from the incubation mixture were used for SDS-PAGE.

Labeling and separation of the protein fragments

Pyridoxal kinase (1.5 M) was incubated with PL-oxime (5×10^{-4} M) and DCC (5×10^{-3} M) in 75 mM potassium phosphate (pH 6.8) at 4°C. The reaction was allowed to proceed for 2 h. Excess of free reagent was removed by gel filtration through Sephadex G-25, equilibrated with the same buffer. The degree of labeling of the enzyme (2.0/dimer) was determined spectrophotometrically using an extinction coefficient of 7.4×10^{3} $cm^{-1} \cdot M^{-1}$ at 340 nm. Pyridoxal kinase labeled with PL-oxime, digested with chymotrypsin for 1 h at a mixing ratio of substrate : chymotrypsin 50 : 1, was chromatographed on a DEAE 5PW column fitted to a Waters HPLC, and the sample eluted with a linear gradient from 0 to 60% B at a flow rate of 1 ml/min [A=5 mM Na-phosphate (pH 7.0), B=0.5 M NaCl in 5 mM Na-phosphate (pH 7.0)]. Fractions collected from the column were tested for fluorescence (excitation wavelength 340 nm) and for absorption at 280 nm. Then, they were lyophilized and examined by SDS-PAGE for characterization of their molecular weights.

Pyridoxal kinase (1.5 μM) was incubated with Ant-ATP (20 μM) in 75 mM potassium phosphate (pH 6.8) at 25°C. Pyridoxal kinase labeled with Ant-ATP was digested with chymotrypsin for 1 h at a mixing ratio (w/w) of substrate:chymotrypsin 50:1. Fifty microlitre of the protein solution was applied to a DEAE 5PW column fitted to a Waters HPLC, and the sample eluted with the above method. Fractions collected from the column were tested for fluorescence (excitation wavelength 460 nm) and for absorption at 280 nm. Then, they were lyophilized and examined by SDS-PAGE for characterization of their molecular weights.

Titration of pyridoxal kinase with Ant-ATP

The fluorescence intensity at 420 nm, as well as the change in emission anisotropy upon excitation at 330 nm, was measured when increasing concentrations of Ant-ATP were added to a fixed concentration of the native and the large fragment (24 kDa) of pyridoxal kinase in 50 mM Tris-HCl, pH 7.5, at 25°C.

The fraction (α) of Ant-ATP bound to enzyme can be calculated from Equation (1):

$$\alpha = \frac{A_{obs} - A_o}{(A_m - A_{obs})\beta + (A_{obs} - A_o)} \quad (1)$$

where A_o and A_m are the emission anisotropy values of free and bound Ant-ATP, whereas Aobs is the observed emission anisotropy of during titration. β is the ratio of fluorescence yields of free and bound Ant-ATP (β=0.5) determined by fluorescence measurements.

The concentration of bound (B) and free Ant-ATP (L), was determined with the aid of Equations (2) and (3):

$$[B] = \alpha[\text{Ant} - \text{ATP}]_{TOTAL} \quad (2)$$

$$[L] = [\text{Ant} - \text{ATP}]_{TOTAL} - [B] \quad (3)$$

The result of titration experiments were fitted to the equations for equivalent and non-equivalent binding sites using the Enzifitter program of R. J. Leatherbarrow, Biosoft.

Spectroscopy

Fluorescence emission spectra were obtained with the use of a Perkin-Elmer Cetus LS-50B spectrofluorometer. The excitation and emission slit widths were set at 2.5 nm. Absorption spectra were recorded with a UVICON-922 spectrophotometer.

Other methods

Protein concentration was determined by the Bradford method (1976). SDS-PAGE was performed on 15% polyacrylamide slab gels according to the method of Laemmli (1970).

Results

Characterization of the binding effects by divalent metal ions

The PL-oxime is a powerful inhibitor of kinase activity even at ATP concentration of 10^{-3}. Fluorescence properties of PL-oxime bound to heavy metal were investigated with Co^{2+}, Zn^{2+}, and Mn^{2+}. Upon addition divalent metal ions to PL-oxime at a final concentration of 0.5 mM, only Co^{2+} resulted in approximately 40% quenching of free PL-oxime (Table 1). While these metal ions (Co^{2+}, Zn^{2+}, and Mn^{2+}) were added to PL, there was no effect on the total fluorescence of pyridoxal (Table 1). From the fluorescence quenching by Co^{2+} as shown in Fig. 1, we determined a dissociation constant of the Co^{2+} to PL-oxime by the method described in (Kim *et al.*, 1988, 1992; Kim and Churchich, 1991), which is a $K_a = 0.7 \times 10^3\ M^{-1}$.

Binding of PL-oxime to the kinase was accompanied by a 3.6 fold decrease in fluorescence intensity (Fig. 2). Co^{2+} at 1 mM has no effect on the fluorescence intensity of PL-oxime bound to the kinase (Fig. 2), suggesting that Co^{2+} is inaccessible to bound PL-oxime. Fluorescence quenching experiments of PL-oxime (5 μM) bound to the kinase (5 μM) were performed in the presence of ATP (0.5 mM). Binding of ATP (0.5 mM) has an effect on the fluorescence intensity of bound PL-oxime. The fluorescence was enhanced by 1.6 fold (Fig. 2). Interestingly, if the PL-oxime remains attached to the enzyme, it does not interact directly with the Co^{2+}

Table 1. Fluorescence quenching effects of PL and PL-oxime by divalent metal ions

Substrate	Divalent metal ions	Fluorescence quenching (%)
PL	Co^{2+}	0
	Zn^{2+}	0
	Mn^{2+}	0
PL-oxime	Co^{2+}	40
	Zn^{2+}	0
	Mn^{2+}	0

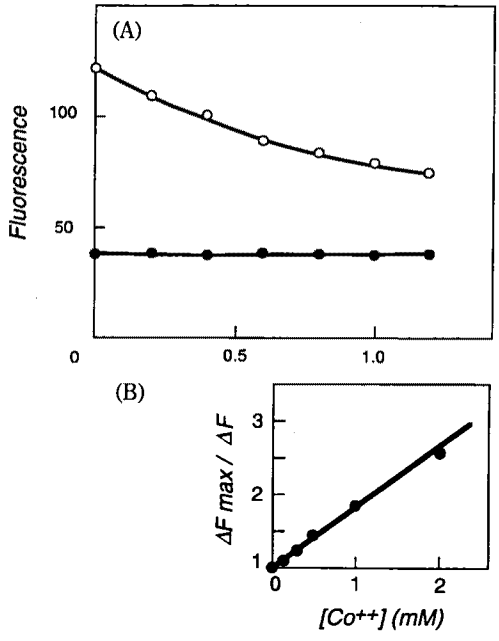

Fig. 1. Titration of the fluorescence quenching effect by Co^{2+} and determination of a dissociation constant (K_A). (A) Changes in fluorescence intensity of upon addition of Co^{2+} to a fixed concentration of PL-oxime (1.5×10^{-5} M) only (○) and PL-oxime (1.5×10^{-5} M) in the presence of pyridoxal kinase (1.5×10^{-5} M) (●) in 70 mM potassium phosphate (pH 7.0) at 25°C. (B) plot of $F_o/F = 1 + K_A [Co^{2+}]$ where F_o is the fluorescence when the enzyme is saturated with ligand and F_o is the measured fluorescence at varying concentrations of ligand. A dissociation constant of 0.7×10^3 M.

atom complexed to ATP. When the binding process is detected in the presence of saturating concentrations of Zn^{2+}-ATP (0.5 mM), characteristic of fluorescence quenching of PL-oxime bound to the kinase gives emission spectra identical to that represented by the Co^{2+} used (data not shown). These results indicate that the binding of PL-oxime to the substrate binding site of the enzyme was not altered by divalent metal ions. From the fluorescence quenching experiments, we can conclude that the PL binding site of the enzyme is distinct from the nucleotide binding site of pyridoxal kinase.

Two structural domains of pyridoxal kinase

Proteolytic digestion of pyridoxal kinase by chymotrypsin generated two fragments of approximately 24 kDa and 16 kDa with concomitant loss of catalytic activity (Fig 9, lane 2). These fragments were separated by DEAE ion exchange HPLC chromatography and used for binding studies with ATP and pyridoxal analogues.

Binding of PL analogue

PL-oxime is a strong competitive inhibitor with respect to the substrate PL of pyridoxal kinase by displacing PL, binds to the folded conformation of the protein with a K_i= 0.1 μM (Churchich and Wu, 1981). The binding process elicits quenching of PL-oxime fluorescence bound to native pyridoxal kinase (Fig. 2 and 5). If cleavage of pyridoxal kinase into two structural domains is restricted to a flexible polypeptide chain connecting two structural domains and the surrounding of the substrate binding sites remains intact, then one should be able to demonstrate binding of substrate analogues to any of the two fragments arising from proteolytic cleavage. In order to determine which structural domain of the pyridoxal kinase is responsible for the substrate PL binding, pyridoxal kinase was digested by chymotrypsin at the ratio of 50 to 1 (w/w) and separated by HPLC equipped with DEAE column as described in the "Materials and Methods". Two proteolytic fragments of 24 kDa and 16 kDa were isolated and binding properties of two fragments were investigated by measuring the fluorescence after incubation with PL-oxime. As shown in Fig. 6, PL-oxime reacted with the large fragment (24 kDa) of the protein, whereas the small fragment corresponding to a molecular mass of 16 kDa did not react with PL-oxime (Fig. 5).

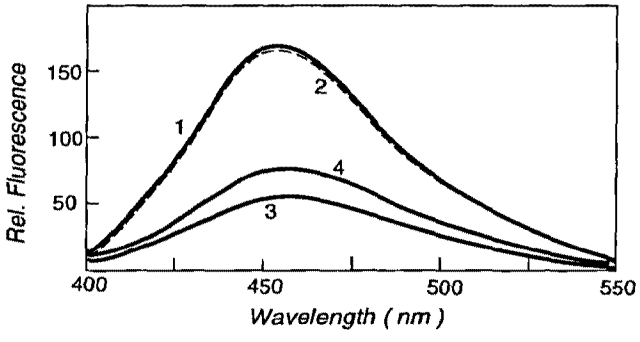

Fig. 2. Fluorescence spectra of pyridoxal-oxime in the absence and presence of proteins, metal ions, and ATP. Emission spectra of PL-oxime (5 μM) in the presence of 1 mM Co^{2+} (curve 1) and PL-oxime (5 μM) in the presence of 1 mM Co^{2+} and 0.5 mM ATP (curve 2). Emission spectra of PL-oxime (5 μM) bound to the kinase (5 μM) in the presence of 1 mM Co^{2+} were performed in the absence (curve 3) and presence (curve 4) of ATP (0.5 mM). A solution PL-oxime (5 μM) + pyridoxal kinase (5 μM) in the absence of 1 mM gives emission spectra identical to that represented by curve 4. Excitation wavelength 360 nm, slits for excitation and emission 2.5 nm.

Affinity labeling of the PL-oxime to PL binding site

PL binding site of the native enzyme was labeled with PL-oxime in the presence of DCC. DCC catalyzes the affinity labeling of PL-oxime to carboxylic group of the acidic amino acids (Kaiser, 1988) as shown in Fig. 3. In the presence of catalyst DCC, the coupling of substrate analog PL-oxime and PL binding site of the catalytic domains of the enzyme has been carried out. Labeling of PL-oxime was monitored by means of fluorescence spectroscopy as shown Fig. 4. As a control experiment, BSA was used for non-reactive protein as a control with PL-oxime.

Covalently-labeled pyridoxal kinase with PL-oxime was incubated with chymotrypsin at the ratio of 50 to 1 (w/w; enzyme to chymotrypsin) for 1 h. At the end of the incubation, the excess reagents were removed by dialysis and separated by a reversed phase column. Fractions collected from the column were tested for fluorescence (excitation wavelength 340 nm) and for absorption at 280 nm. Fractions showing fluorescence were lyophilized and examined by SDS-PAGE for determination of their molecular weights. As shown in Fig. 5, PL-oxime was covalently bound to the large frag-

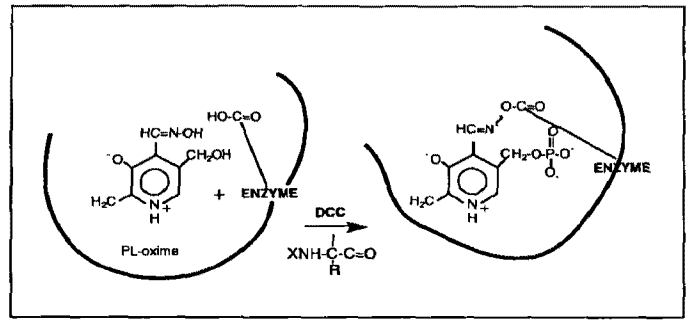

Fig. 3. Reaction scheme represents the chemical coupling of PL-oxime to PL binding site of the proteins.

ment, exhibiting a mobility on SDS-PAGE corresponding to molecular species of 24 kDa, indicating that the large domain was involved in the binding of substrate pyridoxal.

Binding of fluorescent ATP analogs to nucleotide binding site

The substrate analogue Ant-ATP was selected as a probe of nucleotide binding sites because the interaction of Ant-ATP with the nucleotide site is accompanied by an enhancement of the emission covering the wide range of 400~600 nm when excited at 320 nm (Fig. 6). Free Ant-ATP is practically non-fluorescent in aqueous solution at pH 7.0. At a protein concentration of 10 μM and an Ant-ATP concentration of 10 μM, the emission of the anthraniloyl moiety was approximately 3 fold increased when compared to free Ant-ATP at pH 7.0 (Fig. 6). The enhancement of fluorescence revealed that the anthraniloyl moiety bound Ant-ATP is exposed to a non-polar environment. Fluorescence spectra of Ant-ATP bound to the fragment of 24 kDa are practically indistinguishable from those of Ant-ATP complexed to native kinase (Fig. 6). Hence, bound Ant-ATP is highly immobilized with a depolarization factor near unity when complexed to the proteins. In marked contrast to the fragment of 24 kDa, the smaller 16 kDa fragment does not recognize the nucleotide probe Ant-ATP.

Fig. 7A shows the steady-state emission anisotropy values of the Ant-ATP in the presence of protein. The emission anisotropy values were decreased by binding of Ant-ATP to pyridoxal kinase and used to determine the dissociation constant and the stoichiometry of the enzyme-Ant-ATP complex by Scatchard analysis. An

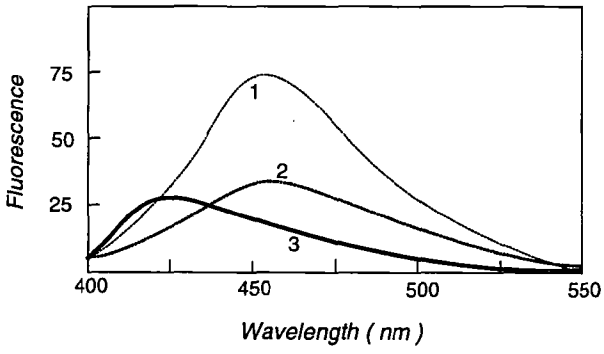

Fig. 4. Analysis of the affinity labeling of PL-oxime to proteins in the presence of DCC. Emission spectra of free PL-oxime (5 μM) only (curve 1). Pyridoxal kinase (5 μM) was incubated with PL-oxime (50 μM), excess PL-oxime was removed by dialysis expensively, and emission spectra (curve 2) of the covalently labeled PL-oxime were measured. As a control protein for nonspecific labeling, 5 μM BSA was reacted with PL-oxime (50 μM), excess PL-oxime was removed by dialysis, and emission spectra (curve 3) were measured. Excitation wavelength 340 nm.

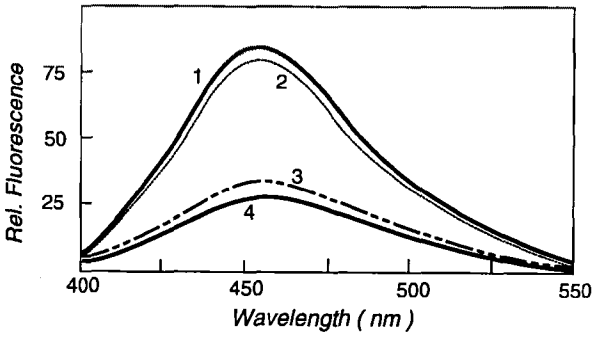

Fig. 5. Fluorescence spectra of proteolytic fragments of pyridoxal kinase reacted with pyridoxal-oxime. Emission spectrum of 1.5×10^{-6} M PL oxime (curve 1). Emission spectra of PL oxime (1.5×10^{-6} M) reacted with 1.5 μM the small fragment of 16 kDa (curve 2) and 1.5 μM the large fragment of 24 kDa (curve 3) after chymotrypsin digestion and separation with HPLC using DEAE column. Curve 4 represents PL oxime (1.5×10^{-6} M) reacted with native 1.5 μM pyridoxal kinase.

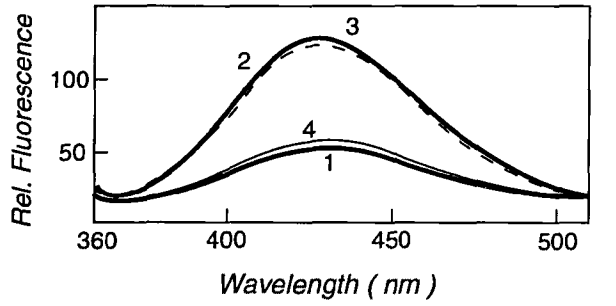

Fig. 6. Binding of Ant-ATP to pyridoxal kinase. Emission spectra of Ant-ATP (5 μM) in the absence (curve 1) and presence (curve 2) of enzyme (10 μM). Ten microliter of two fragments after separation of chymotrypsin-treated kinase with DEAE HPLC chromatography corresponding to molecular weight of 24 kDa (curve 3) and 16 kDa (curve 4) were incubated with Ant-ATP (10 μM), respectively. Excitation was at 330 nm.

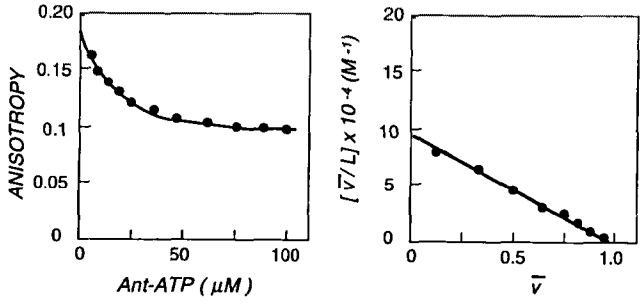

Fig. 7. Titration of pyridoxal kinase with Ant-ATP. (A) Changes in steady-state emission anisotropy upon addition of varying concentrations of Ant-ATP to a fixed concentration of pyridoxal kinase (25 μM). The reaction buffer was in 50 mM Tris-HCl, pH 7.5, at 25°C. The excitation wavelength was 330 nm. Emission was selected by a Corning filter (C-5-3-72). (B) Analysis of the binding results using Scatchard plot of $\bar{v}/[L]$ against $\bar{v}$. A dissociation constant of 13±2 μM for one binding site per molecule of enzyme was obtained.

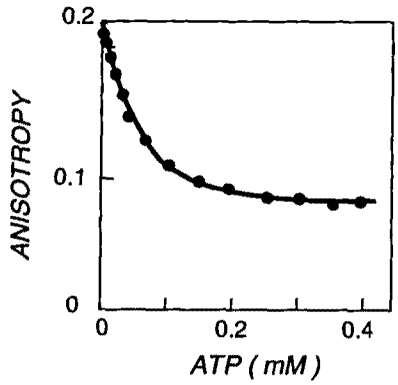

Fig. 8. Displacement of bound Ant-ATP by ATP. A mixture containing pyridoxal kinase (50 μM) and Ant-ATP (10 μM) in 50 mM Tris-HCl, pH 7.5, was allowed to interact with varying concentrations of Zn-ATP for several minutes and the emission anisotropy recorded upon excitation at 330 nm. Measurements were conducted at constant temperature of 25°C.

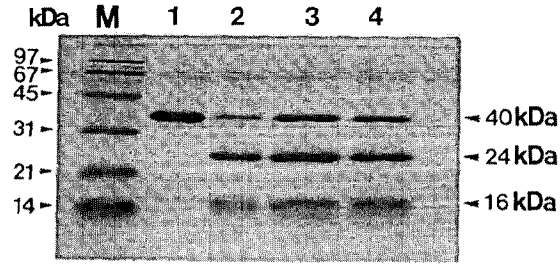

Fig. 9. SDS-PAGE analysis of the protection experiment of pyridoxal kinase against chymotrypsin digestion. The pyridoxal kinase was digested with chymotrypsin at the ratio of 50 to 1(w/w) as indicated. Digestion progress was monitored by 15 % PAGE in the presence of 0.1% SDS. lane M: molecular weight standard proteins; phosphorylase b (97 kDa), bovine serum albumin (67 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa). lane 1: undigested native pyridoxal kinase; lane 2: digested pyridoxal kinase in the absence of substrate analogues; lane 3: digested pyridoxal kinase in the presence of 10 fold excess of PL-oxime; lane 4: digested pyridoxal kinase in the presence of 10 fold excess of Ant-ATP.

analysis of the results included in Fig. 7B by plotting $\bar{\nu}/[L]$ against $\bar{\nu}$ yielded a dissociation constant of 13±2 μM for one binding site (n=1) per molecule of enzyme. If the fluorescent analogue is displaced by ATP, one should be able to detect a progressive decrease in emission anisotropy values as the concentration of ATP increased. Indeed, ATP displaces Ant-ATP from the nucleotide binding site as shown in Fig. 8.

Protection experiment of limited digestion bound to the labeled enzyme

If cleavage of pyridoxal kinase into two structural domains is restricted to a flexible polypeptide chain connecting two structural domains, and if the immediate environment surrounding the substrate binding sites remains intact, then one should be able to demonstrate binding of substrate analogues to any of the two fragments arising from proteolytic cleavage. However, if binding sites of the substrate analogues either PL analogue or nucleotide are located near a flexible hinge region, proteolytic cleavage will be protected from the proteolytic attack. Proteolytic cleavage of the kinase in the presence of the substrate analogues Ant-ATP and PL-oxime was not very much protected (Fig. 9). Judging from the protection experiments, it appears that proteolysis has not perturbed the topography of the nucleotide binding site. These results indicate that both the pyridoxal and the nucleotide binding sites are not located at the hinge region which is susceptible to proteolytic digestion.

Discussion

In this paper we have described the biochemical characterization of pyridoxal kinase using the techniques of limited proteolysis and spectroscopic analysis on the binding properties of heavy metals and substrate analogues, PL-oxime and Ant-ATP. The principal findings reported herein are firstly, that PL-oxime, a strong competitive inhibitor with respect to substrate PL, binds to the catalytic site of the enzyme; secondly, Ant-ATP fluorescent nucleotide analogue binds to the nucleotide site of the kinase.

Fluorescence quenching induced by the binding of PL-oxime to the kinase revealed that the large structural domain is responsible for the binding of substrate pyridoxal. Addition of ATP has an effect on the fluorescence intensity of bound PL-oxime. The fluorescence was enhanced by 1.6 fold. It can be interpreted in two ways: firstly, ATP induces the conformational change that perturbs the increasing of the PL-oxime with the PL binding site; secondly, the PL-oxime is displaced from the enzyme. The second interpretation is less likely because the PL-oxime is a powerful inhibitor of kinase activity even at ATP concentration of 10^{-3}. An interesting aspect of the present studies is the finding that binding of ATP to the kinase perturbs the emission band of the PL-oxime located at the PL binding site. Since it is generally accepted that the substrate pyridoxal and ATP are bound to different physical domains of the protein (Walkers *et al.*, 1991), the simplest interpretation of the fluorescence studies would suggest that binding of ATP to the protein may initiate domain movement and conformational changes might extend to the nucleotide binding site. Heavy metal ions such as Co^{2+} and Zn^{2+} did not disturb the binding characteristics of PL-oxime to the enzyme. Inter-

estingly, if the PL-oxime remains attached to the enzyme, it does not interact directly with the Co^{2+} atom complexed to ATP.

Binding of Ant-ATP to the kinase can be easily detected by fluorescence and emission anisotropy measurements. Ant-ATP recognizes hydrophobic clusters of the nucleotide binding site which become exposed to the solvent as the protein undergoes a structural transition during the catalytic events. This is demonstrated by a progressive decrease in the emission anisotropy of the bound chromophore.

Experimental evidence derived from limited proteolysis and fluorescence spectroscopy support the hypothesis that the substrates are bound to distinct structural domains and that a closed conformation of the enzyme facilitates the phosphorylation of the OH group of pyridoxal.

Both the catalytic site and nucleotide binding site are located in the large structural domain (24 kDa). Pyridoxal kinase is cleaved by chymotrypsin into two fragments of 24 and 16 kDa. An analogue of PL, PL-oxime, binds to the fragment of larger molecular weight. Ant-ATP, a nucleotide analogue, also binds to the large fragment characterized by a molecular weight of 24 kDa.

The most important conclusion derived from spectroscopic and chemical studies involving affinity labeling of the kinase is related to the topography of the catalytic and nucleotide binding site: both are located on the large structural domain of 24 kDa. Proteolytic cleavage of the kinase in the presence of the substrate analogues Ant-ATP and PL-oxime was not preventeded (Fig. 9). Judging from the protection experiments against proteolytic digestion, it appears that limited proteolysis has not perturbed the topography of the nucleotide and PL binding sites of the substrate analogue either PL analogue or nucleotide, indicating the binding sites are not located near to a flexible hinge region which is susceptible to proteolytic attack.

Acknowledgement

This paper was supported by grants from the Basic Science Research Institute Program, Ministry of Education, the Republic of Korea, 1996, Project No. BSRI-96-3434.

References

Bennet, W. S. and Steitz, T. A. (1978) *Proc. Natl. Acad. Sci. USA.* **75**, 4848.

Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248.

Churchich, J. E. and Kim, Y. T. (1989) *Ann. N. Y. Acad. Sci.* **588**, 357.

Churchich, J. E. and Wu, C. (1981) *J. Biol. Chem.* **256**, 780.

Dominici, P., Scholz, G., Kwok, F. and Churchich, J. E. (1988) *J. Biol. Chem.* **263**, 14712.

Hirasuka, T (1983) *Biochim. Biophys. Acta.* **742**, 496.

Kaiser, E. T. (1988) *Angew. Chem. Int. Ed. Engl.* **27**, 913.

Kerry, J. A., Rohde, M. and Kwok, F. (1986) *Eur. J. Biochem.* **158**, 581.

Kim, Y. T. and Churchich, J. E. (1990) *Eur. J. Biochem.* **181**, 397

Kim, Y. T. and Churchich, J. E. (1991) *Biochim. Biophys. Acta.* **1077**, 187.

Kim, Y. T. and Richardson, C. C. (1994) *J. Biol. Chem.* **269**, 5270.

Kim, Y. T. Kwok, F. and Churchich, J. E. (1988) *J. Biol. Chem.* **263**, 13712.

Kim, Y. T., Song, Y. H. and Churchich, J. E. (1996) *Biochim. Biophys. Acta*, in press.

Kim, Y. T., Tabor, S., Bortner, C., Griffith, J. D. and Richardson, C. C. (1992a) *J. Biol. Chem.* **267**, 15022.

Kim, Y. T., Tabor, S., Churchich, J. E. and Richardson, C. C. (1992b). *J. Biol. Chem.* **267**, 15032.

Kwok, F. and Churchich, J. E. (1979) *Eur. J. Biochem.* **93**, 229.

Kwok, F. and Churchich, J. E. (1991) *Eur. J. Biochem.* **199**, 157.

Kwok, F. and Churchich, J. E. (1979) *J. Biol. Chem.* **254**, 6489.

Kwok, F., Scholz, G. and Churchich, J. E. (1987) *Eur. J. Biochem.* **168**, 577.

Laemmli, U. K. (1970) *Nature* **227**, 680.

Loo, Y. H. and Whittaker, V. P. (1967) *J. Neurochem.* **14**, 997.

McCormick, D. B. and Snell, E. E. (1961) *J. Biol. Chem.* **236**, 2085.

McCormick, D. B., Gregory, M. E. and Snell, E. E. (1961) *J. Biol. Chem.* **236**, 2076.

Meister, A. (1990) *Ann. N. Y. Acad. Sci.* **585**, 13.

Neary, J. T. and Diven, W. F. (1970) *J. Biol. Chem.* **245**, 5585.

Neary, J. T., Meneely, R. L., Grever, M. R. and Diven, W. F. (1972) *Arch. Biochem. Biophys.* **151**, 42.

Scholtz, G. and Kwok, F. (1989) *J. Biol. Chem.* **264**, 4318.

Snell, E. E. (1990) *Ann. N. Y. Acad. Sci.* **585**, 1.

Weigard, G. and Reminton, S. J. (1986) *Ann. Rev. Biophys.* **15**, 97.

Wolkers, W. F., Gregory, J. D., Churchich, J. E. and Surpersu, E. H. (1991) *J. Biol. Chem.* **266**, 20761.