To the Graduate Council :

I am submitting herewith a dissertation written by Oh-Shin Kwon entitled "Chemistry of Vitamin $B_6$, Molecular Cloning of its Related Enzymes and Reversible Unfolding Studies of myo-Inositol Monophosphatase with Hsc 70." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy with a major in Biochemistry.

Jorge E. Churchich, Major Professor

We have read this dissertation
and recommend its acceptance :

Accepted for the Council :

Associated Vice Chancellor
and Dean of The Graduate School

# CHEMISTRY OF VITAMIN $B_6$,
# MOLECULAR CLONING OF ITS RELATED ENZYMES
# AND
# REVERSIBLE UNFOLDING STUDIES OF
# *myo*-INOSITOL MONOPHOSPHATASE WITH Hsc 70

**A Dissertation**

**Presented for the**

**Doctor of Philosophy**

**Degree**

**University of Tennessee, Knoxville**

**OH-SHIN KWON**

**December, 1993**

*dedicated to my parents,*

**Young-Do Kwon**

and

**Doo-Yi Lee**

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## ABSTRACT

The main aims of this work are three-fold ; first, to study chemical modification of proteins with vitamin $B_6$ analogues, pyridoxamine or pyridoxamine-5'-phosphate and pyridoxic acid. second, to clone and characterize mammalian genes encoding 4-aminobutyrate aminotransferase (4-aminobutyrate:2-oxoglutarate aminotransferase, E.C. 2.6.1.19) and pyridoxal kinase (ATP : pyridoxal 5'-phosphotransferase, E.C. 2.7.1.35), and third, to study unfolding of myo-inositol monophosphatase and interaction of Hsc 70 with folded and unfolded conformation of the enzyme. In addition, the studies of structual and functional role of pyridoxine (pyridoxamine) 5'-phosphate oxidase (E.C. 1.4.3.5) are also included in appendix.

A general procedure to quantitate the reaction of carbodiimides with carboxyl groups of proteins is described. Pyridoxamine-5'-P reacts with the o-acylisourea intermediate generated during the reaction of carboxyl residues with carbodiimides. The extent of the reaction is determined by measuring the spectroscopic properties, absorption and emission, of P-pyridoxyl residues covalently attached to the proteins. Resolved pig brain aspartate aminotransferase (apoenzyme), inactivated by 1-ethyl-3-(3-dimethylamino propyl) carbodiimide, reacts with [$^3$H] pyridoxamine-5'-P. After trypsin digestion, one peptide labeled with radioactive P-pyridoxyl was separated by reverse phase HPLC.

Luminescence techniques, *i.e.*, fluorescence and phosphorescence, have been employed to study pyridoxic acid bound to proteins through stable amide linkage. Proteins tagged with 4-pyridoxic acid display the following fluorescence properties : (a) emission and excitation spectra centered at around 430 and 320 nm, respectively ; (b) fluorescence quantum yields of 0.3 - 0.4 and (c) average decay times covering the range 8 - 9.6 nanoseconds. The fluorescence properties of the probe have been used to study the dynamics of the protein in the nanosecond time scale. In the absence of molecular

oxygen, free and bound 4-pyridoxic acid exhibit long lived emission at room temperature. The long lived emission is red shifted when compared to fluorescence and decays with average life times ranging from 2.2 to 0.6 milliseconds depending on the nature of the protein. The fluorophore pyridoxic acid covalently linked to proteins is suitable to study the dynamics of proteins, *i.e.*, fast and slow motions of the macromolecule in the nanosecond and millisecond time scales, respectively.

4-Aminobutyrate aminotransferase is a key enzyme of the 4-aminobutyric acid shunt. It is responsible for the conversion of the neurotransmitter 4-aminobutyrate to succinic semialdehyde. By using oligonucleotide probes based on partial amino acid sequence data for the pig brain enzyme, several overlapping cDNA clones of 2.0 - 2.2 kilobases in length have been isolated. The largest cDNA clone was selected for sequence analysis. The amino acid sequence predicted from the cDNA sequence shows that the precursor of 4-aminobutyrate aminotransferase consists of the mature enzyme of 473 amino acids residues and an amino-terminal segment of 27 amino acids attributed to the signal peptide. The cofactor pyridoxal-5'-P is bound to lysine residue 330 of the deduced amino acid sequence of the mature enzyme.

Pyridoxal kinase catalyzes the phosphorylation of vitamin B6 (pyridoxal, pyridoxamine, and pyridoxine) using ATP-Zn as phosphoryl donor. By using oligonucleotide probes based on partial amino acid sequence data for the sheep brain enzyme, several candidate cDNA clones have been isolated from porcine brain cDNA library as well as from sheep liver cDNA library.

*myo*-inositol monophosphatase isolated from pig brain is a very stable dimeric protein characterized by a rotational correlation time of 30 ns. The unfolding and dissociation of the dimeric enzyme (58 kDa) by guanidine hydrochloride have been investigated at equilibrium. The overall process was reversible as judged from the complete recovery of catalytic activity after dilution of guanidine hydrochloride treated samples. Unfolding of *myo*-inositol monophosphatase was monitored by circular

dichroism, fluorescence and steady state emission anisotropy. A folded, monomeric form of the monophosphatase was not detected by the method of denaturant gel filtration. Uncoupled dissociation and unfolding of the oligomeric enzyme could not be demonstrated. The circular dichroism and emission anisotropy results are consistent with a model in which the dimeric protein unfolds in a single cooperative transition from folded dimer to two unfolded monomers.

Fluorescence techniques have been used to investigate the interaction of bovine Hsc 70 with small molecular weight peptides and *myo*-inositol monophosphatase. The emission properties of Hsc 70 remain invariant upon addition of ATP. The results of steady-state fluorescence indicate that the tryptophan residues of Hsc 70 are exposed to the rapidly relaxing aqueous solvent. Binding of RNase S-peptide to Hsc 70 causes protein fluorescence quenching which was used to determine a dissociation constant $k_d$ = 2.7 $u$M for the binary Hsc 70 - (RNase S-peptide) complex. The octapeptide corresponding to the $NH_2$ terminal portion of sickle cell hemoglobin recognizes Hsc 70 and binds with a $k_d$ = 9.3 $u$M. Binding of RNase S-peptide to Hsc 70 produces a small enhancement of ATPase activity. Unfolded *myo*-inositol monophosphatase, tagged with the fluorescent probe EDANS, recognizes Hsc 70 ; the formation of a stable complex was detected by steady-state emission anisotropy measurements. The rate and extent of recovery of catalytic activity of unfolded *myo*-inositol monophosphatase is not influenced by Hsc 70. It is suggested that interaction of Hsc 70 with unfolded proteins in the cell may be able to delay the formation of misfolded structures.

Brain pyridoxine-5'-phosphate oxidase is activated by the tryptophan metabolites 3-hydroxyanthranilate and 3-hydroxykynurenine. 3-hydroxyanthranilate at concentration of 0.03 mM relieves the inhibition elicited by accumulation of the substrate pyridoxine-5'-P ($Ki$ = 60 $u$M). The results of fluorometric measurements indicate that four molecules of 3-hydroxyanthranilate bind to the dimeric enzyme (56 kDa) with an association constant of 5.5 x $10^4$ $M^{-1}$. Differential spectral measurements failed to detect any direct

interaction between the cofactor FMN and the effector 3-hydroxyanthranilate. These results are consistant with the hypothesis that the effector molecules bind to sites of the dimeric protein distrinct from the cofactor site. Limted chymotrypsin digestion of pyridoxine-5'-P oxidase yields catalytically active species that are no longer susceptible to activation by 3-hydroxykynurenine. A polypeptide of 16 kDa containing FMN and endowed with full catalytic activity was isolated by ion-exchange chromatography. It is posturated that the structural domain associated with catalytic activity composes approximately one-half of the molecular mass of pyridoxine-5'-P oxidase (28 kDa), whereas the remaining portion of the macromolecule contains regulatory binding sites.

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## LIST OF ABBREVIATIONS

BSA : bovine serum albumin

CD : circular dichroism

DCCD : dicyclohexyl carbodiimide

DEAE : diethylaminoethyl

EDC : 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide

Fluorescein-PE : (N-fluorescein-5-thiocarbamoyl) dipalmitoyl-L,α-phosphatidyl ethanolamine

FMN : flavin mononucleotide

FPLC : fast protein liquid chromatography

GABA : 4-aminobutyric acid

GABA-T : 4-aminobutyrate aminotransferase

Gu·HCl : guanidine hydrochloride

HEPES : 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid

HPLC : high performance liquid chromatography

Hsc 70 : 70 kDa heat shock cognate protein

1,5-IAEDANS : 5-[2-(2-iodoacetamino) ethylamino] naphthalene-1-sulfonic acid

IMPase : *myo*-inositol monophosphatase

MES : o-morpholino ethanesulfonic acid

Octa peptide : the amino terminal region of the β-chain of sickle cell hemoglobin; Val-His-Leu-Thr-Pro-Val-Glu-Lys

PMP : pyridoxamine-5'-P

PNP : pyridoxine-5'-P

RNase A : ribonuclease A

RNase S-peptide : residues 1-20 of ribonuclease A

SDS-PAGE : sodium dodecyl sulfate-polyacrylamide gel electrophoresis

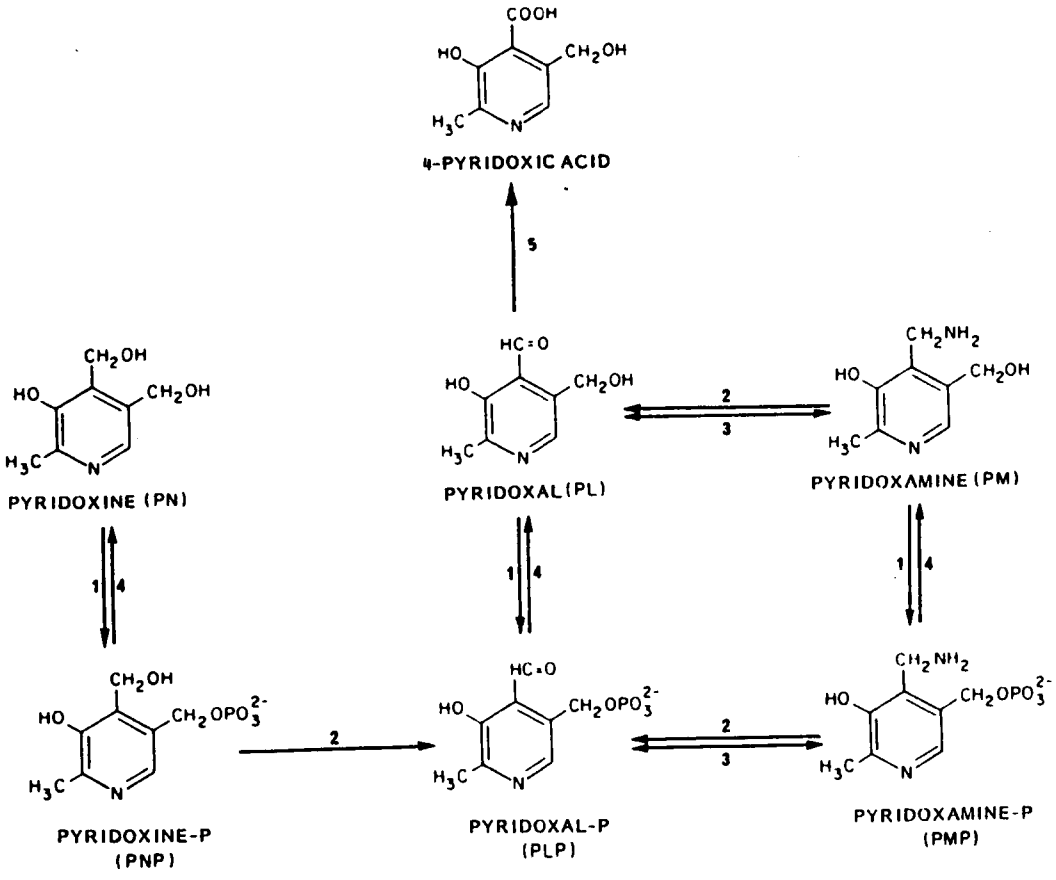
TFA : trifluoroacetic acid

Tris·HCl : tris (hydroxymethyl) aminomethane hydrochloride

# INTRODUCTION

The term "Vitamin $B_6$" was first used by Paul György (1934) to designate an unidentified substance that prevented or cured a florid dermatitis termed *acrodynia.* Vitamin $B_6$ is now used to refer to all 3-hydroxy-2-methy pyridine derivatives that can mimic the biological activity of pyridoxine. Naturally occuring vitamin $B_6$ is present in both free and phospholylated forms. There are six biologically active forms of this vitamin : pyridoxine (PN), pyridoxal (PL), pyridoxamine (PM) and their respective phosphorylated forms, *i.e.*, pyridoxine-5'-phosphate (PNP), pyridoxal-5'-phosphate (PLP), and pyridoxamine-5'-phosphate (PMP). Pyridoxine is distributed throughout the entire plant and animal kingdoms and occures largely in the phosphorylated state (Snell & Haskel, 1971a). Plants synthesize pyridoxine, whereas animals, some bacteria and fungi must obtain it from external sources. In mammalian tissues, the enzymatic conversion compounds, pyridoxal and pyridoxamine, are also present as well as the catalytically active forms of vitamin $B_6$, pyridoxal-5'-phosphate and pyridoxamine-5'-phosphate. The metabolic transformations of vitamin $B_6$ shown in Fig I (for review, Tryfiates et al., 1981; Lumeng & Li, 1980).

In 1944, Gunsalus *et al.* discovered that the active form of vitamin $B_6$ is pyridoxal-P, which is now known to serve as a coenzyme in the deamination, decarboxylation, transamination, racemization, and transsulfuration of aminoacids and in the metabolism of fats and carbohydrates (Snell, 1953). In addition to its catalytic role as a coezyme, vitamin $B_6$ is associated with liver disease, anemias, dental caries and many metabolisms in brain: the tryptophan metabolism, the metabolism of other vitamins and hormones, the development of immune mechanisms, the formation of endogenous oxalates, and the occurrence of uremia.




1 - Pyridoxal kinase

2 - Pyridoxamine-P (pyridoxine-P) oxidase

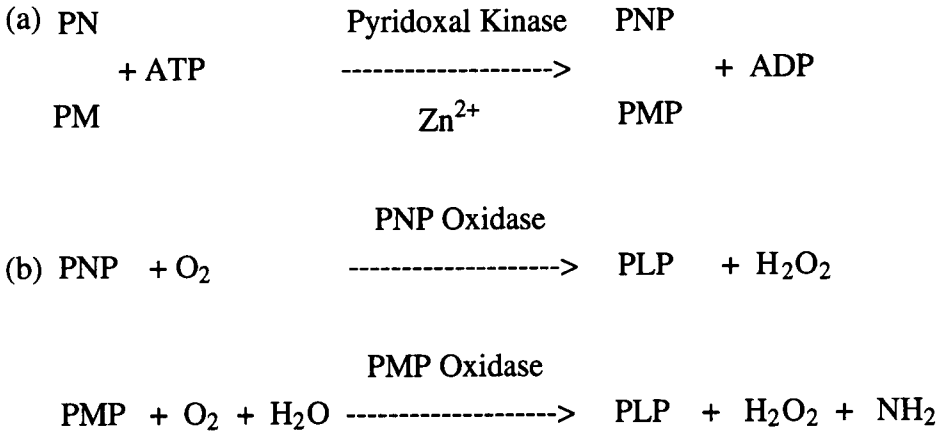
3 - Aminotransferases

4 - Alkaline phosphatases

5 - Aldehyde oxidase and dehydrogenase

**Fig. I.** Pathway of mammalian vitamin $B_6$ metabolism.

The conversion of pyridoxine phophate into pyridoxal phosphate was first demonstrated by Wada *et al.* (1959), who obtained a preparation from rabbit liver that catalysed this reaction. The enzyme pyridoxine-5'-phosphate (PNP) oxidase was later purified from rabbit liver by Morisue *et al.* (1960). Wada & Snell (1961) presented evidence that the oxidation of both pyridoxine phosphate and of pyridoxamine phosphate are catalysed by single protein. It is now known that two steps are involved in the conversion of pyridoxine and pyridoxamine to pyridoxal-5'-phosphate : (a) phosphorylation catalyzed by pyridoxal kinase and (b) oxidation of the phosphorylated vitamins catalyzed by the FMN-dependent pyridoxine (pyridoxamine)-5'-phosphate oxidase.




Pyridoxine (pyridoxamine)-5'-P oxidase (EC 1.4.3.5) was first found in rabbit liver by Pogell (1958) and later studied by many other laboratories including the laboratory of Wada and Snell (1961). The localization of this enzyme in mammals covers a wide range of tissues including liver, kidney, and brain with high activities ; and heart, skeletal muscle, pancreas, and bone marrow with relatively lower activities (Black, 1977). These differences in oxidase activities among different tissues led to the establishment of a complicated network for the pyridoxal-5-P' distribution because tissues with high oxidase activities produce pyridoxal-5-P' not only for internal consumption but also for external supply to other tissues with low oxidase activities.

PNP oxidase, a dimeric flavoprotein with two identical subunits, has molecular weight ranging from 54 to 60 kDa. The flavin mononucleotide (FMN) group acts as a coenzyme and is absolutely required for catalytic activity. The enzymes isolated from different sources have shown that one molecule of FMN binds to one molecule of dimeric enzyme and the coezyme is not covalently bound. The absorption bands of FMN bound to the apoenzyme are shifted from 372 and 445 nm maximum to 380 and 448 nm, respectively.

Kinetic studies have shown that oxidation catalyzed by PNP oxidase proceeds via a binary complex (ping-pong) mechanism with pyridoxine-5'-P and a ternary (sequential) mechanism with pyridoxamine-5'-P. In both cases, oxygen molecules function as the electron acceptor. Results from steady-state kinetics (Choi *et al.*, 1983) showed that parallel Line weaver Burk plots were obtained with oxygen and pyridoxine-5'-P. Choi *et al.* (1983) indicated that the oxidase catalyzed oxidation of pyridoxine-5'-P via 'ping-pong' mechanism because of the observation of competitive inhibition as further evidence.

Since PNP oxidase contributes to the formation of pyridoxal-5'-P, it is evident that some regulatory mechanisms have to exist for adequate formation of pyridoxal-5'-P in cells. Results reported by several laboratories (Snell & Haskell, 1971b; Churchich, 1984; Choi *et al.*, 1987) have shown that the enzyme exhibits product inhibition ; the $K_i$ for the product pyridoxal-5'-P ranges from 1 to 5 uM depending upon the conditions (pH and buffer) of the enzymatic assays. As first suggested by Snell and Haskell (1971b), this process of product inhibition has now been shown to have physiological importance in both liver and brain tissues. Indeed, the low $K_i$ of the enzyme isolated from brain tissues, *i.e.* 2*u*M, is in the range of the concentration of free or loosely bound PLP in brain tissues (Lui *et al.*, 1981). The inhibitory effect exerted by PLP may be one of the mechanisms contributing to the regulation of the concentration of cofactor in brain tissues. In addition, it is possible that accumulation of pyridoxal-5'-P is facilitated by macromolecular

aggregates which restrict the outward diffusion of the metabolites. Kwok and Churchich (1980) used resonance energy transfer and transient kinetic experiments to elucidate the existence of this macromolecular aggregate between pyridoxal kinase and pyridoxine-P oxidase.

The regulation of vitamin $B_6$ levels in tissues is dependent on a number of factors. They include vitamin uptake and metabolism by the liver (Mehansho *et al.*, 1980), uptake mechanisms (Spector & Greenwald, 1978), binding to proteins, and activities of enzyme involved in the metabolism of the vitamin (Snell & Haskell, 1971c; Lumeng & Li, 1975; Meister & Thanassi, 1980). It is apparent that pyridoxal kinase, not only converts pyridoxine, pyridoxal, and pyridoxamine to the phosphorylated forms but also that this enzyme plays a trapping role whereby diffusible nonphosphorylated vitamin forms become intracellularly phosphorylated and poorly diffusible across the cell membrane (Lumeng *et al.*, 1980). In addition, the conversion of PNP and PMP to PLP has been reported to be regulated by product inhibition of PNP oxidase (Merrill *et al.*, 1983). It is apparent that this is the only role played by PNP oxidase since the uptake of pyridoxine in liver cells is dependent on the activity of pyridoxal kinase, but not of the oxidase.

Each tissue maintains an independent pool of vitamin $B_6$ which includes pyridoxine, pyridoxamine, and pyridoxal. The content of different chemical forms of vitamin B6 in this pool is regulated by a combination of enzymes, such as PNP oxidase, pyridoxal kinase, different species of phosphatases, and various PLP binding proteins. Then, the activity of any one of the above enzymes or proteins is also regulated by metabolites from other metabolic pathways. For example, it has been demonstrated that rat liver oxidase is activated by 3-hydroxykynurenine and 3-hydroxyanthranilate both of which are metabolites of tryptophan metabolism (Takeuchi *et al.*, 1985). However, there is no information available on the mechanism underlying the activation process. It is one of the aims of the present work to demonstrate that the modulation of the catalytic behavior of brain PNP oxidase is achieved by the reversible binding of tryptophan

metabolites to regulatory binding sites which are distinct from the catalytic site (Kwon et al. 1991).

Pyridoxal kinase, in the presence of ATP, catalyzes the phosphorylation of pyridoxine, pyridoxamine as well as pyridoxal. Although the enzyme activity is increased in the presence of low concentration of monovalent cation $K^+$, a divalent cation, such as $Zn^{2+}$, $Co^{2+}$, $Mn^{2+}$, $Mg^{2+}$ or $Fe^{2+}$, is essential for the activity. The efficiencies are in the order for the mammalian enzyme, whereas the bacterial enzymes are most active with $Mg^{2+}$.

Pyridoxal kinase is a widely distributed cytosolic enzyme. The activity has been detected in *Escherichia coli* (Hurwitz, 1953), *Streptococcus faecalis* (Gunsalus *et al.*, 1944; Bellamy *et al.*, 1945), yeast (Hurwitz, 1953), and in different mammalian tissues such as skeletal muscle, kidney, liver and brain. The enzyme has been purified to homogeneity from different sources, sheep liver (Karawya & Fonda, 1978) and pig brain (Kwok & Churchich, 1979), human erythrocytes (Chen & Beutler, 1975) and rat brain (Cash et al., 1980).

The pH optimum for activity of the enzyme from mammalian sources is pH 6. The Km values of the pig brain enzyme for pyridoxal, pyridoxine, and pyridoxamine are 25, 17, and 65 uM, respectively (Kwok & Churchich, 1979 ; Gregory, 1980). The Kd for ZnATP is 25 uM (Churchich & Wu, 1981a). The Km values of the sheep liver enzyme for pyridoxal and pyridoxine are 160 and 110 uM, respectively, and Kd for ZnATP is 31 uM. Pyridoxal kinase is not inhibited by pyridoxal phosphate *in vitro* (McCormick *et al.*, 1961). However, many pyridoxal analogs, such as primary oxime, azines and hydrazones of pyridoxal, have been reported to be inhibitors of pyridoxal kinase (McCormick & Snell, 1961; Loo & Whittaker, 1967). Biogenic amines such as dihydroxyphenylalanine (DOPA), dopamine, norepinephrine, tyramine, serotonin, histamine and γ-aminobutyric acid (GABA) play a prominent role in the regulation of pysiological processes. For example, serotonine and dopamine act on smooth muscles

and the cardiovascular system, dopamine is a precursor in the biosynthesis of such neurohormones as adrenaline and noradrenaline, histamine regulates blood pressure and is also connected with allergic and hypersensitivity reactions, and GABA is the inhibitory neurotransmitter in the central nervous system. The content of these biogenic amines in brain has been found to be correlated inversely with the activity of pyridoxal kinase (Ebadi *et al.*, 1968). Since PLP and PMP participate in stages of biogenic amine synthesis and degradation, it has been suggested that the biogenic amines may serve as regulatory role through feedback inhibition of PLP synthesis. Indeed, it has been observed that the biogenic amines inhibit the activity of pyridoxal kinase *in vitro* (Ebadi & Govitrapong, 1979; Abercrombie & Martin, 1980).

Based on steady state kinetic studies, it has been postulated that the phosphoryl group is transferred to pyridoxal with a quaternary complex of enzyme, ATP, pyridoxal and an activating cation (Churchich & Wu, 1982), which are consistent with a rapid equilibrium random Bi-Bi mechanism (Churchich & Wu, 1981b). Affinity labeling studies with adenosine tetraphosphate pyridoxine ($AP_4$-PN) showed that $AP_4$-PN is a competitive inhibitor of the enzyme with respect to both ATP and pyridoxal. However, pyridoxal offered only partial protection against the labeling of the enzyme by adenosine tetraphosphate pyridoxal (Dominici *et al.*, 1988). This suggested that the pyridoxal-binding site may be close to the nucleotide-binding site. Recent NMR studidies of pyridoxal kinase from our laboratory have suggested that the phosphorylation of pyridoxal can occur via direct transfer of the phosphoryl group without formation of a phosphoenzyme intermediate (Wolkers *et al.*, 1991). Because the X-ray structure of this enzyme is still not available although the enzyme has been crystalized recently (Arnone *et al.*, 1989), these results provide important structural and mechanistical information regarding the relative positions of the substrates.

The enzyme isolated from sheep brain is a monomer of 40 kDa that tends to associate into dimers of 80 kDa (Kerry et al., 1986). Limited chymotrypsin digestion

yields two fragments of 24 and 16 kDa with concomitant loss of catalytic activity. Characterization of the two fragments has revealed that the monomer of pyridoxal kinase is cleaved about one third from the carboxyl terminus (Dominici *et al.*, 1989). The 24 kDa fragment generated by proteolysis recognizes both substrate analogues of nucleotide and Pyridoxal (Dominici *et al.*, 1989; Scholz *et al.*, 1990). Although the fuctional role of the small fragment of 16 kDa still remains to be elucidated, it appears more likely that the two-structural domains connected by a flexible polypeptide chain might undergo conformational reorganization prior to the catalytic events like other well studied kinases (Weigan & Remington, 1986; Pineda *et al.*, 1993). More recently, Pineda & Churchich (1993) showed from equilibrium denaturation studies that the nucleotide domain is more stable than the pyridoxal domain of the kinase.

The physiological significance of pyridoxal kinase is not fully known. However, the enzyme has been reported as a key enzyme related to the transport of vitamin $B_6$ (Mulligan & Snell, 1976) as well as vitamin B6 metabolism. The uptake of pyridoxine by facillitated diffusion through the cell membrane is followed by trapping by pyridoxal kinase(Spector & Shikuma, 1978; Spector & Greenwalt, 1978). With growth, kinase activity and PLP content increase in parallel (Bukin, 1976). The specific activities of pyridoxal kinase vary with the amimal species and cell types. For example, liver, kidney, and brain exibit higher activities than lung, heart, skeletal muscle, pancreas, and intestine (McComick et al., 1961). In brain, the activity is highest in the cerebrum and the cerebellar cortex. Ebadi *et al.* (1970) have shown that the daily administration of deoxypyridoxine decreased the concentration of pyridoxal phosphate and increased the activity of pyridoxal kinase in rabbit brain. These results indicated that the tissue availability of pyridoxal phosphate regulates the activity of pyridoxal kinase. The concentration of pyridoxal phosphate in various areas of brain is not uniform, ranging from 0.86 to 1.97 ug/g of tissue (Ebadi & Bifano, 1978). Similarly, the activity of pyridoxal kinase is also unevenly distributed with ranging from 94 to 248 nmol pyridoxal

phosphate formed/g of tissue·hr. With vitamin $B_6$ difficiency, pyridoxal kinase activity in liver decreases as much as 50 %, whereas that in brain decreases only 15 % (Meisler & Thanassi, 1980). Furthermore, such responses were specific to only pyridoxal kinase among the vitamin $B_6$ related enzymes tested such as pyridoxine phosphate oxidase, pyridoxine phosphate phosphatase and pyridoxal kinase. Pyridoxal kinase may serve to protect the pyridoxal phosphate content of brain during periods of vitamin B6 deprivation.

Taken together, these findings are of particular interest because of the intimate relationship of vitamin $B_6$ to brain metabolism. In spite of all these studies, little is still known about the structure of pyridoxal kinase and no sequence is so far available for pyridoxal kinase.

4-aminobutyric acid (GABA), the predominant inhibitory neurotransmitter in many invertebrate systems and in the vertebrate central nervous system (Otsuka et al., 1966; Krnjevic & Schwartz, 1967; Roberts, 1976). GABA mediates neuronal inhibition by binding to the $GABA_A$ receptor and opening an integral chloride channels in the postsynaptic membrane (Olsen, 1982). The subsequent hyperpolarization of the postsynaptic nerve cell is the basis of GABA's inhibitory action (Krnjevic, 1974). Plasma membrane transporters for GABA are widely distributed in the central and peripheral nervous systems (Iverson & Kelly, 1975; Martin, 1976). GABA is sythesized from L-glutamate in brain, various microorganisms, higher plants and other animal tissues by action of glutamate decarboxylase (EC 4.1.1.15) which uses PLP as a cofactor. The formation of GABA is particularly important in the brain since the abnormal levels are associated with a variety of neurological disorders including Huntington's disease (Perry *et al.*, 1973), epilepsy (Tower, 1976), and Parkinsonism (Lloyd *et al.*, 1977). On account of its neural activity, GABA is used for the treatment of epilepsy, cerebral hemorrhage, *etc*.

In addition to its function as a neurotransmitter in nervous tissues, GABA appears to serve as an intermediate in energy metabolism in GABAergic neurons. In synaptic terminals, the regulatory mechanisms must maintain an adequate pool of transmitter GABA in the face of changing levels of neuronal activity. In nonsynaptic regions, GABA presumably functions as a metabolic intermediate, and the regulatory mechanisms should coordinate its turnover with the demands placed on energy metabolism. The apparent metabolic function of GABA arises from the relationship of GABA metabolism to the tricarboxylic acid (TCA) cycle (Baxter, 1970, 1976). The exact proportion of TCA cycle flux that passes through the GABA shunt in GABAergic neurons is unknown. However, regional estimates fall in the range of 1-10 %, and , as would be expected, differ among brain regions and tissues. Once taken up for the neurotansmission, GABA migrates to the mitochondria for its metabolic degradation. The catabolism of GABA proceeds by transamination to succinic semialdehyde and subsequent oxidation to succinate, which is oxidized in the tricarboxylic acid cycle as shown in the Figure II. The transfer of the amino group of GABA to 2-oxoglutamate to form succinic semialdehyde and glutamate, is catalysed by mitochodrial 4-aminobutyrate aminotransferase (GABA-T). Thus, it is not surprising that the concentration of GABA in the brain is controlled by two pyridoxal phosphate dependant enzymes *i.e.* glutamate decarboxylase and GABA-T.

GABA-T is present in several mammalian tissues e.g. in brain, liver, kidney and pancreas (Roberts & Bregoff, 1953; Nikolaeva & Vasil'ev, 1972; Taniguchi *et al.*, 1979), and it has been purified from the brains of several sources including mouse, rat, rabbit, pig and human (Schousboe *et al.*, 1973; Bloch-Tardy *et al.*, 1974; Cash *et al.*, 1974; John & Fowler, 1976; Beeler & Churchich, 1978; Maitre *et al.*, 1975). The enzyme from pig brain is made up of two identical subunits of 50 kDa each (Bloch-Tardy *et al*, 1974; John & Fowler, 1976). The dimeric structure is stable in the pH range 6-8.5, and it does not dissociate into subunits over wide range of protein concentration (0.2 - 4 $u$M) as demonstrated by polarization of fluorescence measurements (Beeler & Churchich, 1978).

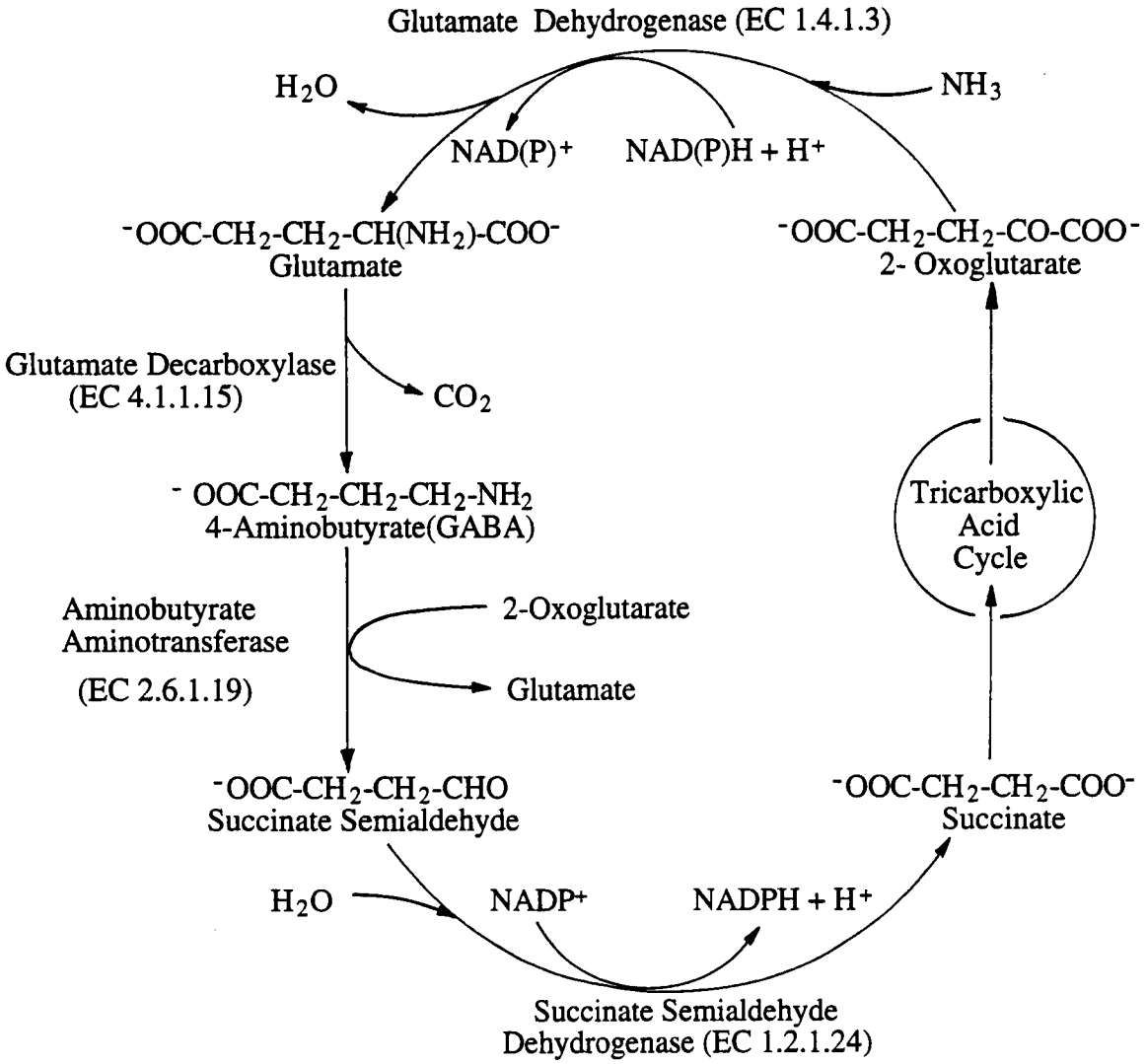



**Fig. II.** 4-Aminobutyrate (GABA) Shunt

Like all other aminotransferase, 4-aminobutyrate aminotransferase is pyridoxal 5'-phosphate dependent. The microenvironment surrounding the molecule of cofactor tightly bound to the native enzyme has been explored with the aid of $^{31}$P-NMR spectroscopy (Felipo, *et al.*, 1983). The results have shown that the chemical shift of the phosphorous signal of the cofactor is pH dependent over the range from pH 6.05 to 7.5. This finding was interpreted to mean that pyridoxal-5'-P is bound in its dianionic form via a rigid salt bridge to positively charged amino acid residues of the active site, leading to considerable lowering of the pK value when compared to free pyridoxal-5'-P in solution (Churchich *et al.*, 1983). Churchich & Moses (1981) reported that the enzyme contains only one coenzyme molecule per dimer with dissociation constant of $10^{-9}$ M. A second molecule of added coenzyme binds much less tightly ($K_d$ = 3 $u$M), but it neither increased activity nor showed any effect on the catalytic parameters when either 4-aminobutyrate or $\beta$-alanine were used as substrates. This unusual behavior might reflect negative cooperative interaction between subunits.

The fungal or mammalian GABA-T catalyzes both the transamination of $\beta$-alanine and 4-aminobutyrate to the same extent whereas the enzyme from bacteria or yeast is inactive for $\beta$-alanine. The activity of GABA-T for $\beta$-alanine is closely related to the occurence of $\omega$-amino acid:pyruvate transaminase ($\omega$-APT) in the organisms. $\omega$-APT activity has been shown for bacteria and yeast (Yonaha *et al.*, 1983; Yonaha *et al.*, 1977), but it could not be detected for fungi and mammals. These observations suggest that the distribution and the substrate specificity of $\omega$-APT and GABA-T were changed at a certain stage of evolution, resulting in alteration of metabolic pathways of $\beta$-alanine and 4-aminobutyrate.

The fundamental catalytic step of transamination is considered to be the loss of hydrogen from the carbon atom bearing the amino group. Kinetic studies have shown that the pig brain and rabbit brain GABA-T conformed to a simple ping-pong binary mechanism, but the published $K_m$ values for GABA and oxoglutarate are lower for the

kidney than for the brain enzyme (Nikolaev & Vasil'ev, 1972 ; John & Fowler, 1976). In the case of pig brain enzyme, the $k_{cat}$ value for GABA:oxoglutarate transamination is 9.6 $sec^{-1}$, and thus a clear rate-limiting step occurs in the GABA:succinic semialdehyde half-reaction (Churchich & Moses, 1981).

Kim & Churchich (1981) have suggested from a set of experiments in which the enzyme was modified with phtalaldehyde that two lysine residues bind the caboxyl groups of dicarboxylic acid. Similar results were reported latter for the enzyme inactivated with bifunctional reagent bis-PLP (Kim & Churchich, 1982). These studies showed that the cofactor of the enzyme is firmly bound to a lysyl residue though a Shiff's base linkage and the bis-PLP blocks lysyl residues that are not involved in the binding of the cofactor, but connected with catalytic activity.

It has been reported that two sulfhydryl residues of the enzyme are critically associated with the catalytic activity (Kim & Churchich, 1983; Choi & Churchich, 1985). It was also shown that iodosobenzoate reversibly oxidizes sulfydryl groups in the dimeric enzyme leading to the formation of intersubunit disulfide bonds with concomitant loss of catalytic acitivity (Kim & Churchich, 1987). The vicinal SH residues are able to participate in the cross-linking reaction because they are positioned in a cleft at the subunit interface, and they are in close proximity, whinin 2 Å of each other. The sulfhydryl residues involved in intersubunit linkage were identified latter by Kim and Churchich (1989).

Since GABA-T is a key enzyme in central nervous system neurotransmitter metabolism, the inhibition of the enzyme by compounds bearing a resemblance to the substrate GABA have received considerable attention over recent years. A number of mechanism-based inhibitors, e.g. aminohexonate (Lippert, *et al.*, 1977) and gabaculine (Rando, 1977), have been studied and tested as specific irreversible inhibitors of the enzyme. The enzyme is also competitively inhibited by mono- and di-carboxylic aliphatic acids, with butyric acid ($K_i$ = 3 mM) being the most effective (Vasil'ev *et al.*, 1970).

Brain 4-aminobutyrate aminotransferase is known to be associated with the mitochodria (Balazs *et al.*, 1966; Salganicoff & DeRobertis, 1965; Van den Berg *et al.*, 1965; Waksman *et al.*, 1968) and on the basis of experiments in which mitochodria were treated with Triton-100, it has been shown that the enzyme is located in the mitochodrial matrix (Salganicoff & DeRobertis, 1965). Further studies demonstrated that the enzyme is associated with the inner membrane of brain mitochondria (Shousboe *et al.*, 1977). A large molecular weight precursor of the enzyme was synthesized in a cell free system (Choi & Churchich, 1986). The precursor was identified by its specific immunoprecipitation with anti-GABA-T antibody and had an apparent molecular weight of 52,000 compared with the mature enzyme with a molecula weight of 50,000. It was also demonstrated that the precursor binds pyridoxal 5'-P and displays catalytic activity. However, little is known about mechanism of its translocation to the mitochondria matrix and the signal sequence for the matrix-targeting.

In general, the matrix-targeting signal is a basic, highly degenerate sequence of 15-35 amino acids that can adopt an amphilphilic $\alpha$-helical comformation (Roise & Schatz, 1988). Perhaps the presequence contains the information not only for the targeting signal destinated to mitochondria, but also for the loss of such signal from the precursor. It is generally assumed that the signal peptide is required to recognize specific binding sites on the mitochondrial membrane and/or to recognize molecular chaperons for efficient translocation, and the matrix-targeting signal is removed by an endoprotease in the matrix. However, several challenging questions remain to be answered : how does the matrix protease recognize the border between the matrix targeting signal and the mature N-terminus, is the signal associated with retardation of folding of the precursor for the transportation and do molecular chaperones recognize this GABA-T precursor ?. In order to further understanding of this translocation process, a detailed analysis of structure and function of the precursor protein and components of the import apparatus must be preceded.

The three dimensional structure of GABA-T is currently under investigation by X-ray crystallography, and a preliminary report of the work has been published (Marcovic-Housley *et al.*, 1990). However, no sequence are available so far for any mammalian 4-aminobutyrate aminotransferase. In order to have further understanding of the transport mechanism to the mitochondria as well as the molecular structure and mechanism of the enzyme, the isolation and sequence of a cDNA encoding the precursor (Kwon *et al.*, 1992) is absolutely necessary. The recombinant DNA technology will make it convenient to obtain large amount of not only the mature form of the enzyme but also the precursor enzyme containing signal peptide. Based on the primary sequence of the cDNA encoding the enzyme, furthermore, the site directed mutagenesis could be carried out to selectively replace individual amino acids for further studies of the enzyme action.

Inositol phosphates are found in the plasma membrane as components of phospholipids, which serves as precursors and storage forms for a number of different messenger molecules. There are three major *myo*-inositol containing lipids, which are ubiquitous components of eukaryotic cells and constitute 2-8 % of the total phospholipid ; these are phosphatidyl inositol (PtdIns), phosphatidyl 4-phosphate [Ptd(4)InsP], and phosphatidyl inosisol 4,5-bisphosphate [Ptd(4,5)Ins$P_2$]. PtdIns is far more abundant (more than 80% of the total phosphatidylinositol), but the three species are in equilibrium (Hokin, 1985; Majerus *et al.*, 1984, 1986).

PtdIns-specific phospholipase C (PLC) enzymes catalyze the hydrolysis of phosphatidyl inositols to produce messenger molecules (Majerus *et al.*, 1986). It is now known that these enzymes hydrolyze not only PtdIns(4,5)$P_2$ but also PtdIns and PtdIns(4)P to yield six water-soluble inositol phosphates and diacylglycerol (Majerus, 1992). The diacylglycerol is a second messenger which promotes the phosphorylation of several proteins by activating protein kinase C (Nishizuka, 1984), and is further hydrolyzed to give arachidonate which may also serve as a cell activator (Majerus *et al.*, 1986).

One of the major signal transducing pathways is that in response to extracellular signals of hormones or neurotransmitters, inositol-1,4,5-trisphosphate ($InsP_3$) is rapidly released from phosphatidyl inositol-4,5-bisphosphate in the plasma membrane by a variety of membrane receptors (Berridge & Irvine, 1989; Putney *et al.*, 1990). $InsP_3$ appears to be the active species responsible for the release of a second messenger $Ca^{2+}$ ions from intracellular stores (Berridge, 1987).

*myo*-Inositol has six hydroxyl groups that can be substituted with phosphate. There are 66 different possible myo-inositol phosphates including the three known inositol cyclic phosphates. Even though many other inositol phosphates are formed in cells, little is known about the functions of these other compounds. The complexity of the pathways for formation and degradation of these compounds implies that inositol phosphates other than $InsP_3$ may also function as second messengers.

$InsP_3$ is soon dephosphorylated, in three dephosphorylation steps, to myo-inositol for the resynthesis of phosphatidyl inositol. The last of these reactions, the hydrolysis of *myo*-inositol-1-P to *myo*-inositol, is catalyzed by *myo*-inositol monophosphatase (Hallcher & Sherman, 1980; Berridge, 1989). Furthermore, the *de novo* synthesis of *myo*-inositol from glucose-6-phosphate yields *myo*-inositol-1-P, rather than free *myo*-inositol. Thus, inhibition of *myo*-inositol monophosphatase interupts the metabolism pathway of inositol phosphates, and prevents formation of phosphatidyl inositol.

*myo*-Inositol monophosphatase (EC 3.1.3.25) was first studied in yeast by Chen and Charalampous (1966) who found that this enzyme is a component of the pathway for the biosynthesis of *myo*-inositol from glucose-6-P via the intermediate *myo*-inositol-1-P. The enzyme has been purified from a number of different sources including rat (Takimoto *et al.*, 1985), bovine brain (Gee *et al.*, 1988; Attwood *et al.*, 1988; Meek *et al.*, 1988; ) and even from lily pollen (Gumber, 1984). In all cases, the enzyme has a molecular weight ranging between 55 to 60 kDa, and is composed of two identical subunits.

In 1971, an interesting result was reported by Allison and Stewart : the acute administration of lithium, a drug widely used to treat manic depressive illness, results in a 30% decrease in the level of *myo*-inositol in rat cerebral cortex. Allison *et al.* (1976) have showed that lithium also causes the level of *myo*-inositol-1-P to increase in the same tissues. Furthermore, the therapeutic concentrations (0.5 to 1.5 mM) of lithium inhibits *myo*-inositol monophosphatase uncompetitively with an apparent $K_i$ of 0.8 uM (Hallcher and Sherman, 1980). These observations implicate that inositol monophosphates is the most plausible target for the action of lithium as a therapeutic agent (Berridge *et al.*, 1989).

*myo*-Inositol monophosphatase was initially designated as inositol-1-phosphatase. However, since it hydrolyzes all inositol monophosphates (except Ins 2-P) with approximately equal catalytic efficiencies, its name has been changed to inositol monophosphatase (Inhorn & Majerus, 1988; Ackermann et al., 1987). Unlike the other inositol phosphate phosphatases, this enzyme has relatively poor affinity for inositol phosphates ($K_m$~$10^{-4}$M), and is also capable of hydrolyzing a number of non-inositol-containing substrates including 2'-AMP, 2'-GMP, NADP, and $\beta$-glycerol phosphate, *etc.* The enzyme requires $Mg^{2+}$ as a cofactor with $K_m$ of 1mM, but other divalent ions, *i.e.*, $Ca^{2+}$ and $Mn^{2+}$, are competitive inhibitors of $Mg^{2+}$ binding with $K_i$ values of 18 $u$M and 2 $u$M, respectively (Hallcher & Sherman, 1980).

In view of its potential importance as a drug target, the action mechanism of *myo*-inositol monophosphatase is of considerable interest. Shute *et al.* (1988) have shown that $Li^+$ traps a phosphoryl enzyme intermediate preventing nucleophillic attack by water. This result suggested that myo-inositol phosphatase operates a ping-pong mechanism whereby phosphate is transferred from the substrate to the enzyme and then from the enzyme to water; lithium cation inhibits the second step. On the other hand, Ganzhorn & Chanal (1990) proposed a direct attack on the phosphorous by water. Recently, it has been shown from isotope exchange experiments that the enzyme works through a ternary

complex with substrate and water (Baker and Gani, 1991). In either mechanism, and active site residue may be involved in catalysis, acting as a nucleophile or a general base catalyst. The pH dependence of myo-inositol monophosphatase suggests that two groups with pK values around 6.5 must be deprotonated for activity, and proton inventories indicate a single proton is transferred in the transition state of the reaction (Ganzhorn & Chanal, 1990). More recently, chemical modification studies have provided evidence that histidine residues are important for such a catalytic residue and proton acceptor (Pelton & Ganzhorn, 1992). Although these observations are consistent with general base catalysis by histidine (Schowen, 1977), none of these results can clearly exclude the existence of a phosphorylenzyme intermediate. Therefore, detailed kinetic studies are desirable to provide additional information on the mechanism of catalysis.

*myo*-Inositol monophosphatase is a key cytosolic enzyme in the inositol phospholipid signaling system. The complete c-DNA and deduced primary amino acid sequence of the bovine enzyme has been reported (diehl *et al.*, 1990). Recently, c-DNAs encoding for the functional homologues from human and rat have been reported, and noted to be practically all identical in structure and function (McAllister *et al.*, 1992). Furthermore, the crystal structure of human inositol monophosphatase, a dimer of identical 277 amino acid subunits (30 kDa, McAllister *et al.*, 1992) has been determined to 2.1 Å resolution (Bone *et al.*, 1992). The enzyme exists a dimer of identical subunits, each folded into a five-layered sandwich of three pairs of $\alpha$-helices and two $\beta$-sheets. Despite these studies, little is known about the conformational stability of the enzyme. The structue and energetics, in principle, are closely related to how the protein function. Thus, one of the aims of present work is to investigate the stability of the inositol monophosphatase in the presence of denaturating agents (Kwon *et al.*, 1993).

From the denaturation studies, several information about a protein can be obtained (Pace, 1986). First of all, it generally provides an estimation of the conformational stability, $\Delta G_D^{H20}$, of the protein, i.e., how much more stable the globular and native

conformation of the protein is than unfolded, denatured conformations. SThe conformational stability of a protein is dependent on the steepness (m) of the denaturation transition as well as the denaturation midpoint (Pace, 1986). The conformational stability of almost all naturally accurring globular proteins is between 5 and 15 kcal $mol^{-1}$ (21-63 kJ $mol^{-1}$). It appears to be adventageous to living organisms to have proteins for which the folded, biologically active conformation is only marginally more stable than unfolded, inactive conformations. The reasons for this are still not clear, but it may have to do with the protein's turnover or function (Arfin & Bradshaw, 1988; Beckel & Schellman, 1987). Denaturation curves are especially good for measuring the differences in conformational stability among proteins differing slightly in chemical structure because of differences in amino acid sequence or alterations resulting from chemical modification. Second, even though equilibrium measurements are made, it is possible to draw deductions about the mechanism of folding of the protein, *e.g.*, it can be shown that unfolding does not follow a two-state mechanism. Finally, inferences can be drawn about the structure of the protein, *e.g.*, when the denaturation curve has more than one step, it generally indicates that the protein has more than one domain and it is possible to estimate the relative stabilities of the domains.

Most of the physical properties change substantially when a protein unfolds. Consequently, many techniques can be used to follow unfolding; those used most often are ultraviolet difference spectroscopy, circular dichroism, optical rotation, fluorescence, and NMR. Urea and GdnHCl are used for a variety of purposes by protein chemists. They offer several advantages over other means of unfolding a protein such as acid , heat, or detergents. First, the product is better defined because the degree of unfolding is maximized. Proteins in 8M urea or 6M GdnHCl with their disulfide bonds broken approach a randomly coiled conformation. Second, unfolding is more likely to approach a two-state mechanism. And, third, denaturation is more likely to be completely reversible.

These features are especially important in attempting to estimate the conformational stability of a protein.

Refolding denatured polypeptide chains *in vitro* has been an important issue for the understanding of protein folding pathways. Until recently, it has been assumed that protein folding and assembly occurs spontaneously in cells since many proteins refold after denaturation with urea, guanidine HCl, pH, or heat treatments. The self assembly hypothesis which states that all the information required to determine the final conformation of protein can reside in the amino-acid sequence itself has been derived from the classic work of Anfinsen (1973) on the refolding of ribonuclease *in vitro*. This spontaneous process is driven by small differences in the Gibbs free energy between the unfolded and native states. Thus this process requires neither further input of energy nor any steric information extrinsic to the polypeptide itself. However, this hypothesis is contrary to a simple knowledge that efficient folding *in vivo* promarily requires antifolding activity to prevent protein folding during sythesis or across membrane. Furthermore, refolding *in vitro* is frequently very inefficient in comparison to folding *in vivo*, and often requires protein concentrations and physicochemical conditions very different from these occuring intracellularly. Many proteins exist in cells as homo- or hetero-oligomeric complexes that in some cases are assembled before folding of the individual polypeptide chains is complete. Indeed, diverse studies of protein folding, unfolding and translocation across membranes suggest that in cells families of abundant proteins modulate protein folding, assembly and disassembly, and facilitate in the degradation of malfolded polypeptides.

When cells are exposed to external stress, such as a sudden increase in temperature, a class of different proteins, known as the heat shock (stress) proteins or commonly referred to as molecular chaperons, are rapidly expressed. The external stress that activates the synthesis of stress protein has in common the potential to cause the intracellular accumulation of denatured or aberrant protein (Munro & Pelham, 1985). The

term "molecular chaperon" initially referred to a nuclear protein nucleoplasm which binds to histones and facilitates ordered nucleosome assembly in the nucleus by preventing the formation of improper protein aggregates (Laskey *et al.*, 1978; Dingwall & Laskey, 1990). This term now applies generally to a family of proteins that mediate the correct assembly of other polypeptides, but that are not components of the functional assembly (Ellis & Hemmingsen, 1989). Known molecular chaperons do not convey steric information for either polypeptide folding or oligomerization, so the principle of self-assembly is not violated in this sense.

The majority of the currently identified chaperons belong to three highly conserved protein families which are widely distributed from prokaryotes to plants and mammals (reviewed in Gething & Sambrook, 1992; Ellis & van der Vies, 1991; Craig & Gross, 1991; Hartl *et al.*, 1992). The first class is represented by the chaperonin families (groEL/hsp 60). The second family includes the 70 kDa heat shock proteins (Hsp 70) and the heat shock cognate proteins (Hsc 70) which are constitutively expressed in the cell cytoplasm under normal conditions. The last class is known as cytosolic Hsp 90 proteins that have apparent $M_r$'s varying from 87 to 92kDa. Molecular chaperon function, in general, is not as catalysts of secondary structure formation, but rather to recognize and stabilize partially folded intermediates during polypeptide folding, assembly and disassembly. A number of studies have shown that Hsp 70 family participate in the stabilization or generation of unfolded protein precursors before assembly in the cytosol (Beckmann *et al.*, 1990) or translocation into organelles including the ER and mitochodria (Chirico *et al.*, 1988; Deshaies *et al.*, 1988; Murakami *et al.*, 1988), in stabilization of newly translocated polypeptides before folding and assembly (Gething *et al.*, 1986; Bole *et al.*, 1986; Pelham, 1986; Kang *et al.*, 1990), in rearrangement of protein oligomers (Georgopoulos *et al.*, 1990; Rothman & Schmid, 1986; Deluca-Flaherty *et al.*, 1990), in dissociation of protein aggregates (Pelham, 1986; Skowyra *et al.*, 1990), and in the degradation of rapidly turned-over cytosolic proteins (Chiang *et al.*, 1989; Dice,

1990). Despite the differences in their sequences and oligomeric structures, the general features of interaction of Hsp 60 proteins with their target polypeptides is very similar to that of Hsp 70. Chaperonin 60 proteins bind unfolded precursors before export of secretory proteins (Bochkareva *et al.*, 1988; Lecker *et al.*, 1989) or assembly of protein oligomers (Georgopoulos & Ang, 1990) in *E. coli* and help in the folding and assembly of polypeptides translocated into chloroplasts or mitochondria in eukaryotic cells (Hartl & Neupert, 1990). Hsp 90 proteins seem to stabilize a variety of target proteins in an inactive or unassembled state (Craig, 1988). The hsp 90 proteins bind ATP in a cation-dependent manner and undergo autophosphorylation (Csermely & Kahn, 1991). In contrast to Hsc 70 and chaperonin, Hsp 90 proteins do not seems to catalyze the hydrolysis of ATP.

In eukaryotes, the hsp 70 gene is a member of a gene family which is highly conserved in evolution and contains both heat inducible and non-inducible genes (Craig, 1988; craig & Gross, 1991). Over recent years, it has been widely assumed that the Hsp 70 families have several structural and functional features in common : (a) they consist of a highly conserved amino-terminal ATP binding domain followed by a more variable carboxy-terminal substrate binding domain (Chappell *et al.*, 1987; Flynn *et al.*, 1989; Flaherty *et al.*, 1990; Normington *et al.*, 1989), (b) they bind to unfolded or partially denatured polypeptides (Palleros *et al.*, 1991; Flynn *et al.*, 1991), and (c) in many cases, they utilize the energy of ATP hydrolysis to release the bound substrates ( Flynn *et al*, 1989; Liberek *et al.*, 1988).

The majority of the Hsp 70 family members are expressed constitutively, and genetic studies suggest that many of these proteins are essential for cell viability even under non-stress conditions of growth (Craig, 1988; Georgopoulos, 1990). The constitutively expressed Hsc 70 has been originally found as a clathrin uncoating ATPase by Rothman and his collaborators (Schmid *et al.*, 1984; Schlossman *et al.*, 1984; Braell *et al.*, 1984). Coated vesicles, which are intermediates in the pathway of receptor-mediated

endocytosis, are covered in a cagelike structure formed by the association of times of the protein clathrin. In the presence of ATP, hsc 70 binds to clathrin cages and is induced to hydrolyze the ATP. This process results in the distruption of clathrin-clathrin interactions and ultimately causes dissociation of the cage into clathrin trimers. However, it has been assumed that the ATPase must have other functions because the proteins are expressed far more than would appear to be necessary; it comprises about 1 % of total cellular protein which is about a 30 fold molar excess over clathrin, and also because it binds to nuclei after heat shock. It seemes very similar to a general properties of the hsp 70 family. Thus, It was realized that this protein is a constitutively expressed member of the stress-70 family (Hsc 70) (Chappell *et al.*, 1986).

Even in unstressed cells, it is now known that hsc 70 plays an important role in the translocation of certain proteins across organellar membranes (Deshaies *et al.*, 1988; Chirico *et al.*, 1988). In addition, hsc 70 binds to calmodulin (Stevenson & Calderwood, 1990), to apocytochrome *C* (Sadis *et al.*, 1990), and to several hydrophilic peptides (Flynn *et al.*, 1989). A portion of hsc 70 is also found associated with microtubules (Green & Liem, 1989) and with the avian progesterone receptor (Kost *et al.*, 1989). The use of antisera specific for the 70 kDa stress proteins precipitates a variety of as yet unidentified cellular proteins, some of which associate in a cell-cycle specific manner (Milarski *et al.*, 1989). In many cases, the addition of ATP to complexes formed between hsc 70 proteins and other polypeptide causes distruption of the complex (Palleros *et al.*, 1991; Greene & Eisenberg, 1990; Flynn *et al.*, 1989; Rothman & Schmid, 1986). These observations suggest that as a member of chaperones, hsc 70 associates with misfolded proteins that accumulate during stress and further assist certain protein folding and trafficking pathways for nascent polypeptides in non-stressed cells (Pelham, 1986; Rothman, 1989, Fischer & Schmid, 1990; Beckmann *et al.*, 1990).

Proteolytic digestion of the bovine Hsc 70 protein yields a 44 kDa N-terminal fragment that retains ATPase activity but can not bind clathrin (Chappell *et al.*, 1987).

This fragment has been crystallized, and its three dimensional structure has been reported (Flaherty *et al.*, 1990). The atomic structure of the Hsc 70 fragment is strikingly similar to that of rabbit skeletal muscle actin (Kabsch *et al.*, 1990; Flaherty *et al.*, 1991). In addition, the nucleotide binding domains of the protein have been shown to closely resemble the substrate binding domain of hexokinase (Steitz *et al.*, 1976; Flaherty *et al.*, 1991).

Although it is now clear that hsp 70 protein plays an essential role in both normal cellular processes and in the heat stressed cells, several important questions still remain to be answered about the mechanism of action and the in vivo specific function and substrates of the protein. For example, how can a single heat protein associate with many other polypeptides that lack extensive sequence similarity but may share similar conformational or structural features ? What is the biochemical mechanism that couples ATP hydrolysis to peptide binding and release, and how does this result in a molecular chaperone activity ? Does Hsc 70 need any accessory proteins for regulatory modifications in the activity ? Thus, for better understanding of the heat shock protein-polypeptide ligand interaction, more biophysical studies of the protein structure are neccessary.

The main purposes of this work are three-fold ; first, to study the chemical modification of proteins with vitamin $B_6$ analogues, pyridoxamine or pyridoxamine phosphate and pyridoxic acid, second, to clone and characterize mammalian genes encoding 4-aminobutyrate aminotransferase and pyridoxal kinase, and third, to study the unfolding of *myo*-inositol monophosphatase and interaction of Hsc 70 with folded and unfolded conformation of the enzyme. In addition, the studies of structual and functional role of pyridoxine (pyridoxamine)-5'-phosphate oxidase which were presented when I was in the master progam are included in the appendix.

# PART I

# CHEMICAL MODIFICATION OF PROTEINS WITH VITAMIN $B_6$

# CHAPTER 1

# REACTION OF PYRIDOXAMINE-5'-P WITH CARBOXYL RESIDUES OF PROTEINS

## INTRODUCTION

The carboxyl groups of proteins (aspartic and glutamic acid) have been found to be implicated in specific carbodiimide protein interactions (Solioz, 1984). The *o*-acylisourea intermediate formed during the reaction of carboxyl residues with carbodiimide can react with a nearby amino group of an amino acid chain to yield inter and intramolecular crosslinked protein species (Pennington & Fisher, 1981). Moreover, a rearrangement reaction of the *o*-acylisourea intermediate can lead to the formation of a stable N-acylurea adduct (Borders *et al.*, 1989). The reaction of the o-acylisourea intermediate with radiolabeled nucleophiles ([$^{14}$C] glycine ethyl ester) has been used to quantitate the extent of the chemical modification of carboxyl residues (Matsuo *et al.*, 1980).

The aim of the present work is to demonstrate that a spectroscopic probe (pyridoxamine-5'-P, or pyridoxamine) can be used to quantitate the extent of the interaction of the protein with carbodiimide reagents. Pyridoxamine-5'-P, a cofactor of aspartate aminotransferase, reacts with the *o*-acylisourea intermediate formed during the reaction of carboxyl residues of the protein with carbodiimide. The modified peptide has been isolated.

## EXPERIMENTAL PROCEDURES

### Labeling of Proteins

Reactions were carried out in 25 mM o-morpholino ethanesulfonic acid (MES), pH 6, containing 5 mg/ml of protein, 10 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and 1 mM pyridoxamine-5'-P, or 10 mM pyridoxamine. The reaction mixture was incubated at 25 °C for 3 hours. At the end of the reaction, the modified proteins were passed through Sephadex-G-25 column, precipitated with 70 % acetone (v/v) and dialysed against 0.1 M phosphate buffer (pH 7).

The reaction of resolved aspartate aminotransferase with the carbodiimide reagent was carried out as described above with the exception that tritiated pyridoxamine-5'-P (10 mCi/mmol) was used in the incubation reaction. The modified enzyme was treated with guanidinium-HCl (6M), dialysed against 50 mM ammonium bicarbonate (pH 8.6) and digested with trypsin (mixing molar ratio, substrate : trypsin, 50 : 1) at 37 °C for 5 hours. The digested sample was lyophilized dissolved in 0.1 % TFA and applied to a vydac C18 column (25 x 0.46 cm) for reverse-phase HPLC. The peptides were eluted using a linear gradient from 5 to 50 % of eluant B in 90 minutes. Eluant A: 0.1 % TFA; eluant B: 0.1 % TFA in 80 : 20 acetonitrile / $H_2O$. The fractions were lyophilized, dissolved in the scintillation mixture and counted in a scintillation counter (Beckman, LS 7,500).

The degree of pyridoxamine-5'-P incorporation in the modified proteins was determined by measuring the absorbance at 325 nm ($\varepsilon_{325}$ = 9,000 $M^{-1}cm^{-1}$) or by diluting the protein solution and determining the fluorescence at 395 nm (excitation at 325 nm) using P-pyridoxyl-acetyl as standard of known quantum yield (q = 0.08). P-pyridoxal-acetyl was prepared as described in reference (Higgins & Wilson Miles, 1978).

Inactivation of Aspartate Aminotransferase

It was studied at pH 6 and pH 6.5, using a concentration of resolved enzyme of 0.5 mg/ml, EDC concentration of 10 mM and pyridoxamine-5'-P concentration of 1 mM in 25 mM MES. Aliquots withdrawn from the incubation mixture at several time intervals were mixed with pyridoxal-5'-P (1mM) incubated for 25 minutes at 25 °C prior to enzymatic assays. A control of holotransaminase (pyridoxal-5'-P form) was treated with EDC and pyridoxamine-5'-P under identical experimental conditions and assayed for catalytic activity.

Preparation of Aspartate Aminotransferase

The method used in the purification of aspartate aminotransferase from pig brain is based on methods (Jenkins & Sizer, 1960; Martinez-Carrion *et al.*, 1967) previously used in the purification of the enzyme from pig heart. The enzyme was resolved into apoenzyme and free cofactor, and enzymatic assays were conducted using a coupled enzymatic system as described by Churchich *et al.*, (1969). Protein concentration was determined by the calorimetric method of Lowry *et al.* (1951).

Synthesis of [$^3$H] Pyridoxamine-5'-P

[4',4',$^3$H] pyridoxamine-5'-P was synthesized by tritium labeled sodium borohydride (340 mCi/mmol) reduction at room temperature of the imine formed by pyridoxal-5'-P in $NH_4OH$. The compound was purified through chromatography on an amberlite-CG-50 column ($H^+$ form) (1x30 cm) eluted with water and 0.01 HCl.

Spectroscopy

Fluorescence measurements were conducted in a precision fluorimeter equipped with two Bausch and Lomb monochromators and a light source (Xenon lamp, 150 watt). Absorption spectra were recorded in a Shimadzu, UV-160 spectrophotometer.

Fluorescence decay measurements were made using the monophotonic technique with an Ortec Nanosecond spectrometer. Excitation was set at 330 nm and the emission filtered through a glass filter (Corning, CS-3-72). The fluorescence decay function was deconvoluted (O'Connor & Phillips, 1984).

Materials

$NaB[^3H]_4$ (340 mCi/mmol) was purchased from Du Pont New England Nuclear. Pyridoxamine-5-P, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and dicyclohexyl-carbodiimide (DCCD) from Sigma. The proteins bovine serum albumin and lysozyme from Boehringer. Resins used in chromatography were purchased from Biorad and Pharmacia.

## RESULTS AND DISCUSSION

Derivatization of protein carboxyl groups by carbodiimides is efficient at acid pH in the region 5.0 - 6.5, but still proceeds at a reasonable rate at neutral pH. Incubation of bovine serum albumin and lysozyme with carbodiimide and pyridoxamine-5'-P at pH 6 resulted in the incorporation of P-pyridoxyl residues as indicated by the spectroscopic data included in Table 1-1. The incorporation of approximately 2 P-pyridoxyl residues/mol of lysozyme resulted in 40 % loss of catalytic activity. Pyridoxyl-5'-P bound to the proteins displays an emission band centered at 395 nm (excitation at 325 nm) as shown in Fig. 1-1.

The fluorescence emitted by the P-pyridoxyl residues of aspartate aminotransferase decays in a multiexponential manner with an average lifetime of approximately 3 nanoseconds (Fig. 1-2). The polarization of fluorescence values obtained by excitation at 330 nm are influenced by the size of the macromolecule to which the

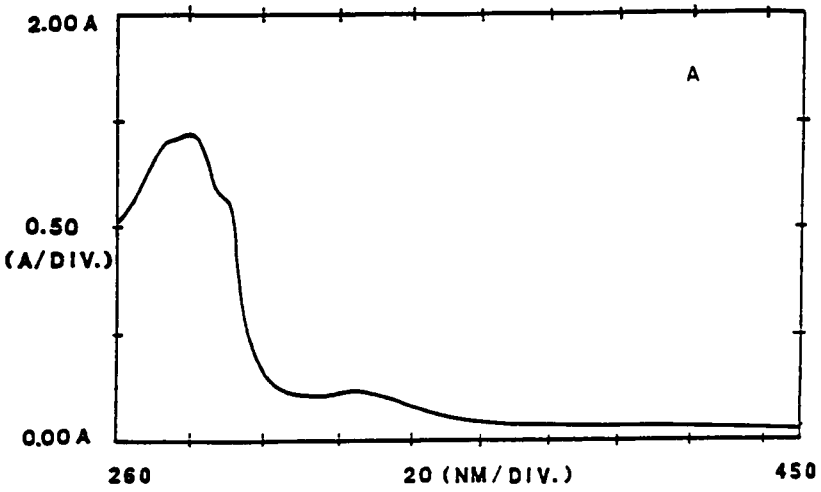
**Table 1-1**

*Spectroscopic Properties of PM bound to Proteins*

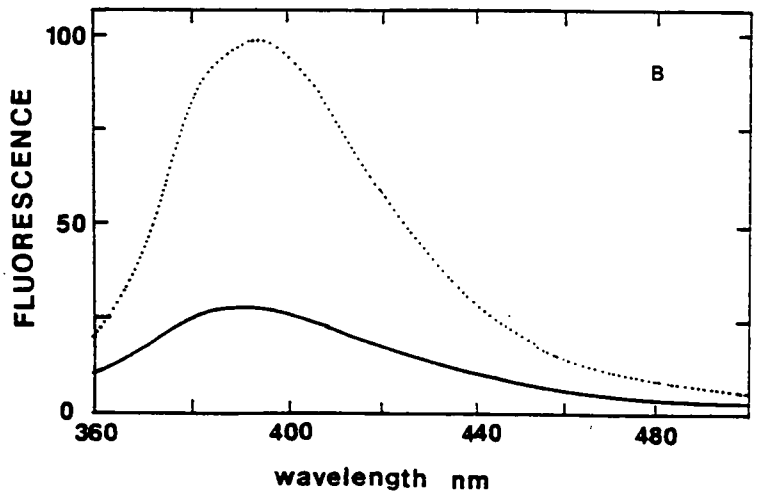
| Sample | $\lambda_f$ (nm) | $\tau_f$(ns) | P | Degree of Labeling (mol/mol)* | Activity (%) |
|---|---|---|---|---|---|
| BSA | 395 | 3.0 | 0.3 | 2.0 | - |
| Lysozyme | 395 | 3.2 | 0.18 | 1.9 | 60 |
| Apo GOT | 395 | 3.0 | 0.3 | 1.8 | 2 |
| Apo GABA-T | 395 | - | - | 1.9 | 60 |

Excitation wavelength at 325 nm. $\lambda_f$ is the maximum wavelength of fluorescence emission, $\tau_f$ is fluorescence lifetime, P is polarization of fluorescence.

* mol/dimer for Apotransaminases




- *Absorption Spectrum*




- *Fluorescence Spectra of PM Free & Bound*

**Fig. 1-1.** *(A) Absorption spectrum of lysozyme modified with pyridoxamine.* A degree of labeling of 1.8 moles PM / mol enzyme was detected by absorption spectroscopy. The enzyme is partially inactivated (40 %).

*(B) Fluorescence spectra of pyridoxamine free(····) and bound to lysozyme(–) at pH 6.0.* The excitation wavelength was 325 nm.

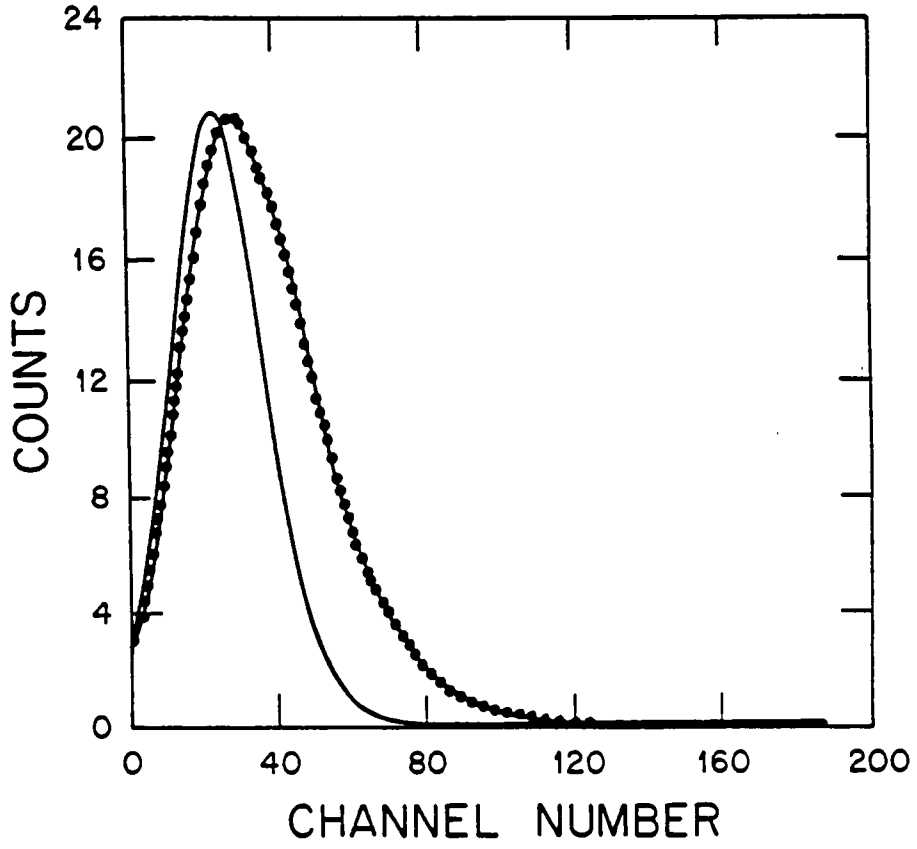



**Fig. 1-2.** *Fluorescence decay of P-pyridoxyl residues bound to aspartate aminotransferase (•) and lamp profile (-).*

The deconvoluted decay with fitted to a biexponential curve. $\alpha_1 = 0.99$, $\tau_1 = 3$ ns, $\alpha_2 = 0.01$, $\tau_2 = 18$ ns.

fluorescent residues are covalently attached. Aspartate aminotransferase and bovine serum albumin of molecular masses ranging between 94 and 68 kDa exhibit polarization of 0.3, whereas lysozyme (16 kDa) is characterized by a polarization value of 0.18.

Inactivation of Resolved Aminotransferase

Inactivation of resolved aminotransferase by EDC takes place at pH 6.5 and 5 as shown in Fig. 1-3. At the lowest pH value, the half life for inactivation is 50 minutes. The inhibitory effect exerted by chemical modification of the protein is completely prevented when pyridoxal -5'-P is bound to the aminotransferase, indicating that carbodiimide reacts with aminoacid residues located in the catalytic site. A potential side reaction during modification of the carboxyl groups of proteins by carbodiimides is covalent crosslinking between adjacent subunits. To establish whether such intersubunit crosslinking had occurred, resolved aspartate aminotransferase modified by EDC was subjected to SDS-polyacrylamide gel electrophoresis. The modified protein migrated as a single band of 48 kDa that was indistinguishable from the control, untreated enzyme. Therefore, there was no evidence that intersubunit crosslinking had occurred during inactivation of the aminotransferase.

Another potential side reaction taking place during modification of carboxyl groups by carbodiimide is the formation of stable N-acylisourea adducts. To ascertain whether the formation of stable N-acylisourea adducts had taken place, the resolved aminotransferase was treated with 5mM DCCD, dicyclohexyl [$^{14}$C] carbodiimide, and tested for incorporation of radiolabel. Although the resolved enzyme was inactivated by DCCD, there was little incorporation of radiolabeled ligand (0.2 mol/dimer) in the modified enzyme after dialysis against 25 mM MES (pH 6).

Thus, most of the *o*-acylisourea intermediate generated by reaction with carbodiimides are trapped by the nucleophilic group of pyridoxamine-5'-P. Scheme 1-1 shows a possible reaction mechanism for the modification.

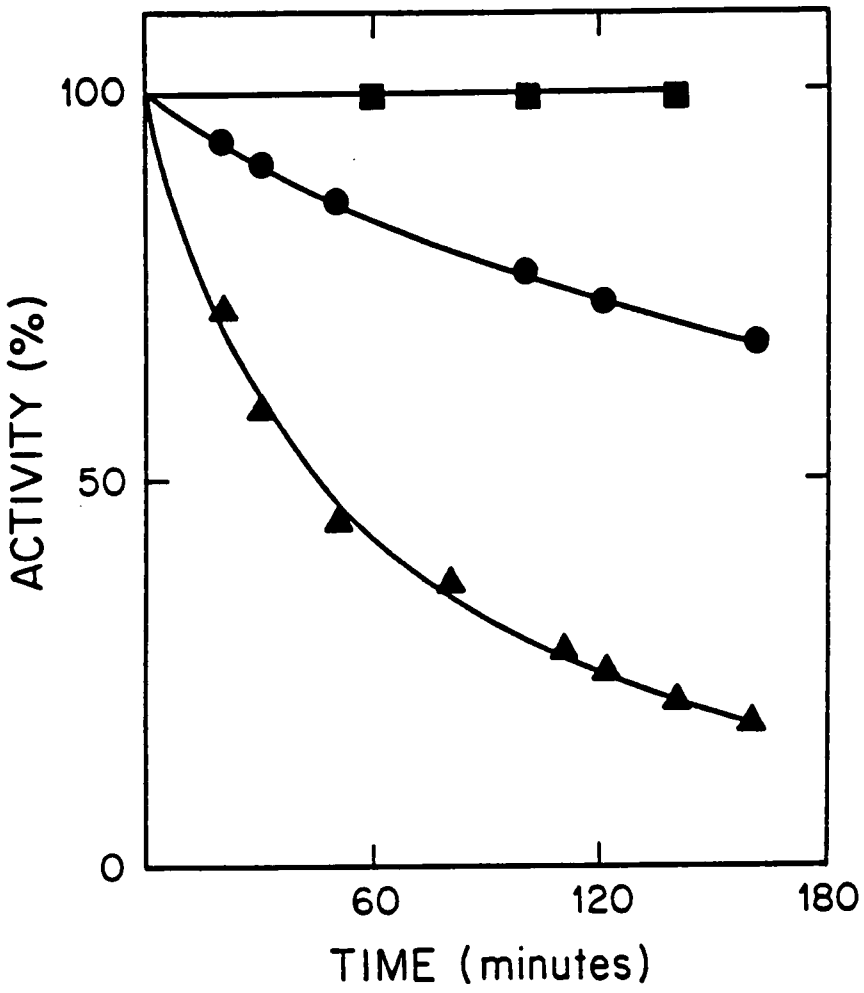
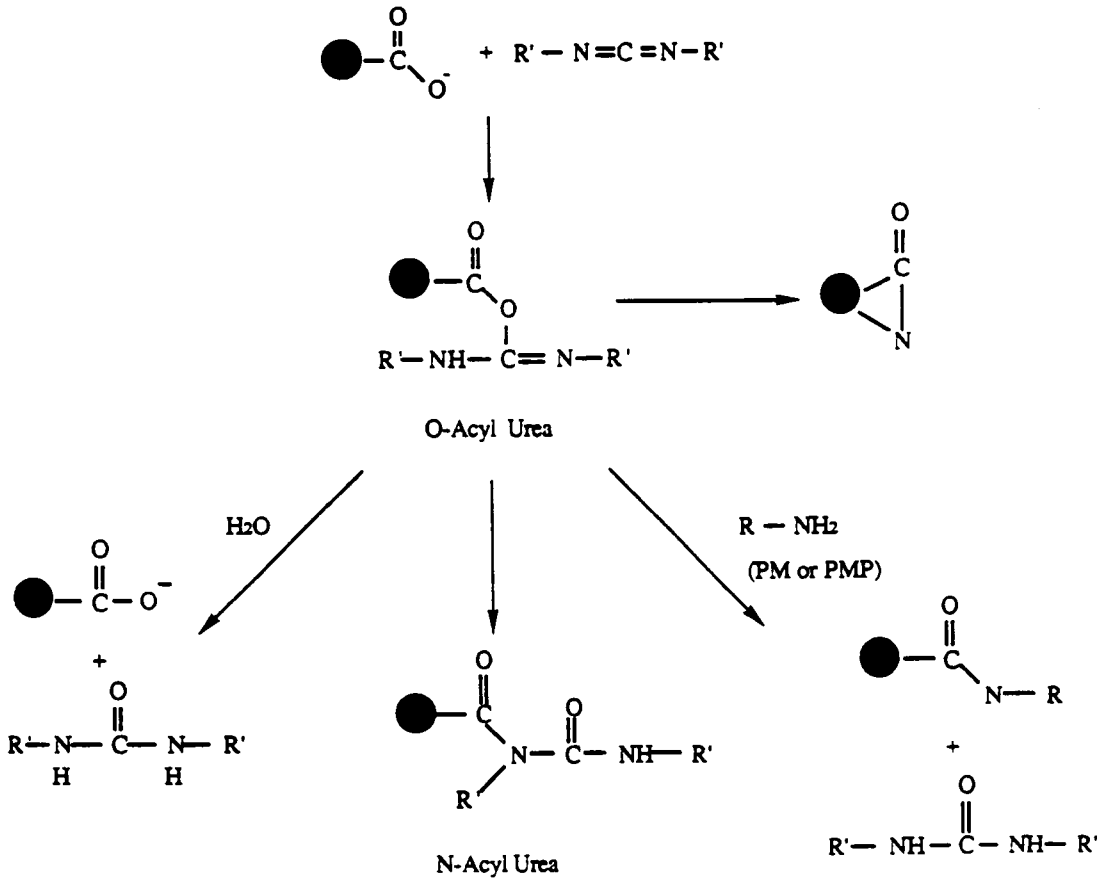



**Fig. 1-3.** *Inactivation of resolved aspartate aminotransferase by EDC at pH 6.5 (•) and pH 6 ( ▲) in 25 mM MES buffer.*

0.5 mg/ml of resolved enzyme was incubated in the presence of EDC (10 mM) and pyridoxamine-5'-P (1mM) at 25 °C. Aliquots withdrawn at the indicated times were mixed with pyridoxal-5'-P (1 mM), incubated for 15 minutes and assayed for catalytic activity. Results obtained with the holoenzyme (PLP form) incubated with EDC and pyridoxamine-5'-P under similar experimental conditions are included (■).




**SCHEME 1-1**

Possible reaction mechanism for the interaction of PM or PMP with a carboxyl group of protein activated by EDC.

Identification of modified peptides

To ascertain whether the loss of catalytic activity produced by reaction of EDC could be related to modification of few carboxyl residues of the aminotransferase, resolved enzyme (5mg) reacted with EDC and tritiated pyridoxamine-5'-P (10 mCi/mmol) was digested with trypsin an the peptides separated by reverse-phase HPLC. Upon separation by reverse phase chromatography, only one radioactive peptide was detected. A typical elution profile obtained with the digested aminotransferase is shown in Fig. 1-4.

Automated Edman degradation was performed to identify the modified peptide. The following amino acid sequence was obtained for the radioactive peptide :

FLFPFFXSAYQGFASGNLEK

x indicates the position of the radiolabeled amino acid. This sequence overlaps a segment of 20 amino acids, positions 216-235 of the entire primary sequence of cytosolic aspartate aminotransferase from pig brain (Christen *et al.*, 1985). Accordingly, the modified amino acid (x) corresponds to aspartic acid 222. In this respect, it should be noted that aspartic acid 222 is involved in either binding pyridoxal-5'-P or assisting pyridoxal catalysis (Jansonius et al., 1985). The same experimental approach could be used to investigate the interaction of carbodiimides with GABA transferaminase.

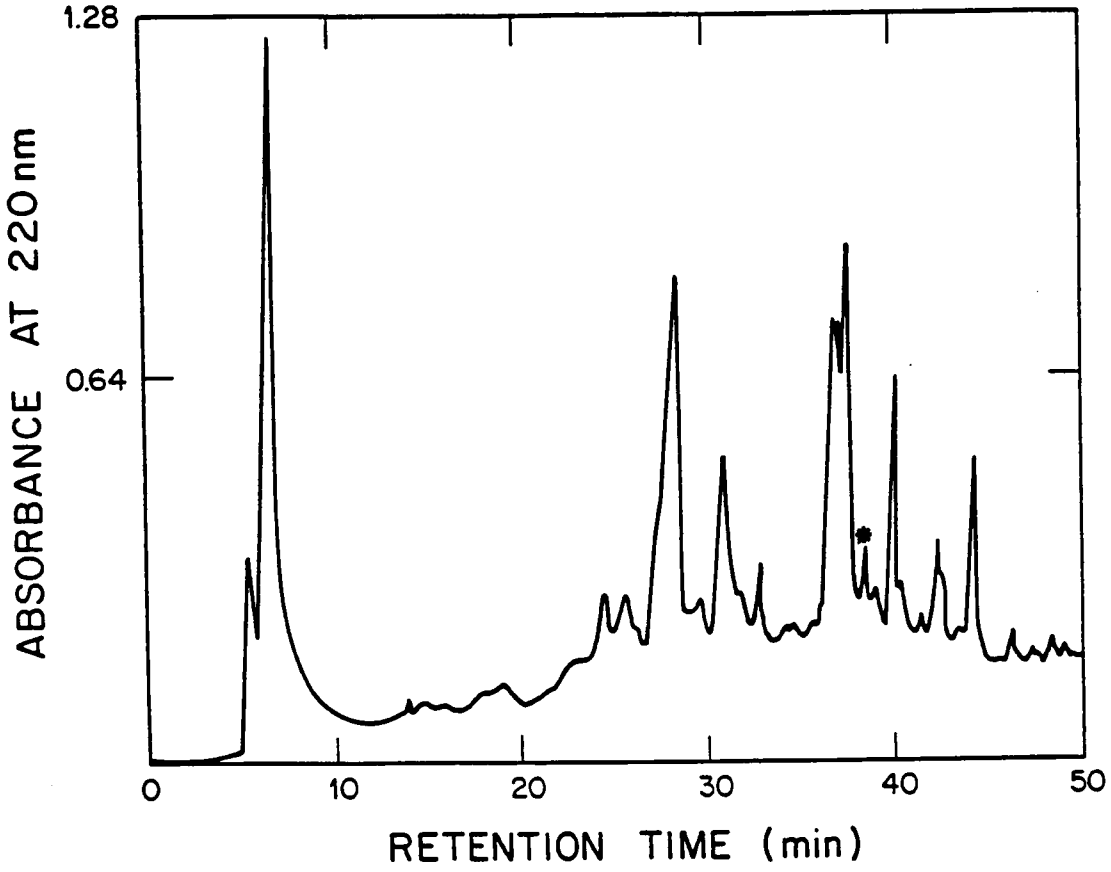



**Fig. 1-4.** *Separation of tryptic peptides from modified aspartate aminotransferase by reverse-phase HPLC.*

The separation was performed with a linear gradient from 5 to 60 % B eluant in 90 minutes at a flow rate of 0.8 ml/min (eluant A = 0.1 % TFA, eluant B = 0.1 % TFA in 80 : 20 acetonitrile / $H_2O$). Elution was monitored at 220 nm. Fractions of 0.5 ml were collected and 10 $\mu$l of each fraction were withdrawn for counting radioactivity. The fraction indicated in the figure (*) contains 3,000 cpm.

Amino acid sequence for the radioactive peptide : FLFPFFXSAYQGFASGNLEK

X indicates the position of the radiolabeled amino acid.

# CHAPTER 2

## LUMINESCENCE SPECTROSCOPY OF PYRIDOXIC ACID BOUND TO PROTEINS

### INTRODUCTION

4-Pyridoxic-5'-P, has the ability of forming stable complexes with aminotransferases (Churchich, 1972). The spectral changes detected when the ligand is bound to apoproteins were used to investigate the micro environment of the cofactor binding sites.

It is the purpose of the present work to examine the spectroscopic properties of 4-pyridoxic acid covalently attached to proteins and to investigate the possibility of using this fluorophore as a probe of the rotational and diffusional dynamics of proteins in solution. Pyridoxic acid, the final product of the catabolism of pyridoxal-5'-P (Snell, 1990), reacts with nucleophilic groups of proteins; particularly lysyl residues. The fluorophore, attached to the protein through a stable amide linkage, exhibits fluorescence and long lived emission properties at room temperature suitable to study slow and fast motion of proteins.

Since the 4-pyridoxic acid is smaller than most of the extrinsic probes commonly used in labeling macromolecules, it is anticipated that they would produce minor conformational changes when reacted with nucleophilic groups of enzymes.

# EXPERIMENTAL PROCEDURES

## Materials

Pyridoxic acid, N,N'-carbonyldiimidazole, cytidine 2',3'-cyclic monophosphate, RNase A, bovine serum albumin (BSA) and polylysine were purchased from Sigma. Sephadex G-25 was obtained from Pharmacia.

## Labeling of Proteins

It is known that carboxylic groups react with N,N'-carboxyldiimidazole to give acylimidazole intermediates (Paul & Anderson, 1960). Pyridoxic acid (10 mM) and N,N'-carbonyldiimidazole (20 mM) dissolved in 1 ml of anhydrous N,N'-dimethylformamide were stirred 30 minutes at 25 °C. Then, 0.2 ml of the reaction mixture was added to 2 ml of a protein solution (100 *u*M) in 0.1 M Hepes buffer (pH 8) and allowed to react at 37 °C for 3 hours.

The modified protein was separated from excess reagent by dialysis against 0.1 M Hepes (pH 7) followed by gel filtration through a sephadex G-25 column equilibrated with 0.1 M Hepes (pH 7). The degree of labeling was determined by absorption measurements at 318 nm using an extinction coefficient of 7800 $M^{-1}cm^{-1}$. Protein concentration was determined by Bradford's method (Bradford, 1976). Similar procedure was applied for labeling polylysine (MW 100,000), except that the polymer was dissolved in buffers (pH 7) containing 0.5 M NaCl.

Enzymatic activity of RNase A was determined with 2',3'-cyclic cytidine monophosphate as substrate (Cook *et al.*, 1960). Assays were performed at 25 °C in 0.1M Tris-HCl (pH 7) containing 0.1 M NaCl and the reaction was monitored at 284 nm. Absorption spectra were recorded in a Shimadzu UV 160 spectrophotometer.

Fluorescence and Phosphorescence Spectroscopy

Polarization of fluorescence measurements were done in a SLM polarization apparatus. Illumination, provided by a Xenon lamp (150 W), was selected by a grating monochrometer. Fluorescence polarized light emitted by the samples was passed through Corning filters (C-S-3-72, C-S-3-69).

Fluorescence decay measurements were made using single photon counting in an Ortec model 8200 nanosecond spectrometer. A free running flash lamp operating in air at 1 atm. was used as excitation source. The lamp was pulsed at 10 kHz. The emission was filtered through a glass filter (Corning C-S-3-72). The decay function were deconvoluted and fitted to either a mono or a biexponential decay using non-linear least square analysis (O'connor & Phillips, 1984).

Fluorescence and phosphorescence spectra were recorded in a Perkin Elmer LS-50B spectrofluorometer. Several methods were used to remove oxygen from the cuvettes prior to phosphorescence measurements at room temperature. A quartz fluorescence cuvette equipped with a stopcock (Hellma) was used in these experiments. The $N_2$ purging method basically involves bubbling of the sample through a small glass capillary followed by immediate capping of the cell. Bubbling for the order of 15 minutes is usually sufficient for removal of molecular oxygen. For removal of oxygen from buffer diluents, the solutions were stirred under vacuum, then bubbled with nitrogen for 30 minutes. For complete deoxygenation, glucose oxidase (0.4 $u$M), glucose (0.3 %) and catalase (0.05 $u$M) were added to the sample containing cuvette, which was then stirred and capped with a Teflon stopper (Calhoun *et al.*, 1983).

# RESULTS

## Fluorescence Properties of the Modified Proteins

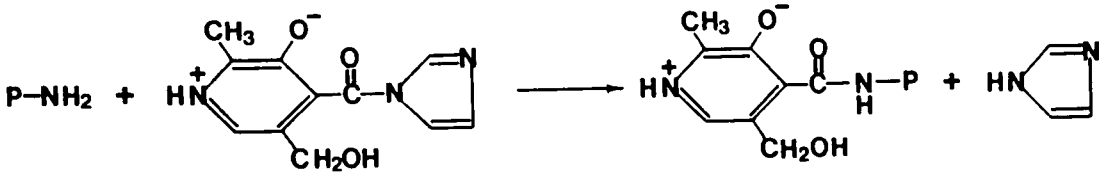
Pyridoxic acid reacts with N,N'-carbonyl diimidazole to form an acylimidazole intermediate. In the presence of proteins, the intermediate reacts with lysyl groups to yield stable amides as shown in Scheme 2-1.

The incorporation of approximately 4 mol of pyridoxic acid per mol of protein was observed when bovine serum albumin was pre incubated with 10 fold molar excess of the acylimidazole intermediate under the conditions described in "Experimental Procedures". The degree of labeling is decreased to approximately 1 mol of pyridoxic acid per mol of protein when the molar mixing ratio of acylimidazole to protein is only two fold.

Polylysine reacts with activated pyridoxic acid and a degree of labeling of approximately 8 mol of pyridoxic per mol of polymer is easily attained after 1 hour incubation at room temperature.

Ribonuclease A, on the other hand, reacts with the acylimidazole intermediate to yield a modified protein devoid of catalytic activity when the stoichiometry of binding is only 1 mol of pyridoxic acid per mol of the protein.

Table 2-1 lists the fluorescence properties of proteins tagged with 4-pyridoxic acid, where it can be seen that the fluorophore exhibits spectroscopic properties similar to those of free pyridoxic acid. A strong emission band covering the spectral range 330 - 500 nm is characterized by a fluorescence quantum yield ranging from 0.3 to 0.4. The fluorescence emitted by the covalently bound fluorophore decays in a multiexponential manner with lifetime values ranging from $<\tau> = 8$ to $<\tau> = 10$ ns. A typical deconvoluted fluorescence decay is shown in Fig. 2-1.




**SCHEME 2-1**

Table 2-1

*Spectroscopic Properties of 4-pyridoxic acid and 4-pyridoxic-Protein*

| Sample | $\lambda_f$ (nm) | $Q_f$ | P | $k_q \cdot 10^{-9}$ ($M^{-1}sec^{-1}$) | $\tau_f$ (ns) | $\lambda_p$ (nm) | $\tau_p$ (ms) |
|---|---|---|---|---|---|---|---|
| pyridoxic acid | 430 | 0.40 | 0.03 | 2.10* | 8.0 | 490 | 0.9 |
| BSA | 430 | 0.41 | 0.24 | 0.45 | 8.8 | 480 | 2.2 |
| RNase | 430 | 0.28 | 0.10 | 1.20 | 8.0 | 470 | 0.8 |
| Polylysine | 430 | 0.35 | 0.05 | - | 9.8 | 490 | 0.6 |

All parameters were determined with excitation wavelength of 320 nm at room temperature.

$\lambda_f$ is the maximum wavelength of fluorescence emission, $Q_f$ is the quantum yield of fluorescence, P is polarization of fluorescence, $\tau_f$ is fluorescence lifetime, $k_q$ is the bimolecular rate constants of dynamic fluorescence quenching, $\lambda_p$ is the maximum wavelength of phosphorescence and $\tau_p$ is phosphorescence lifetime.

* Pyridoxic lactone was used for $K_q$ parameter

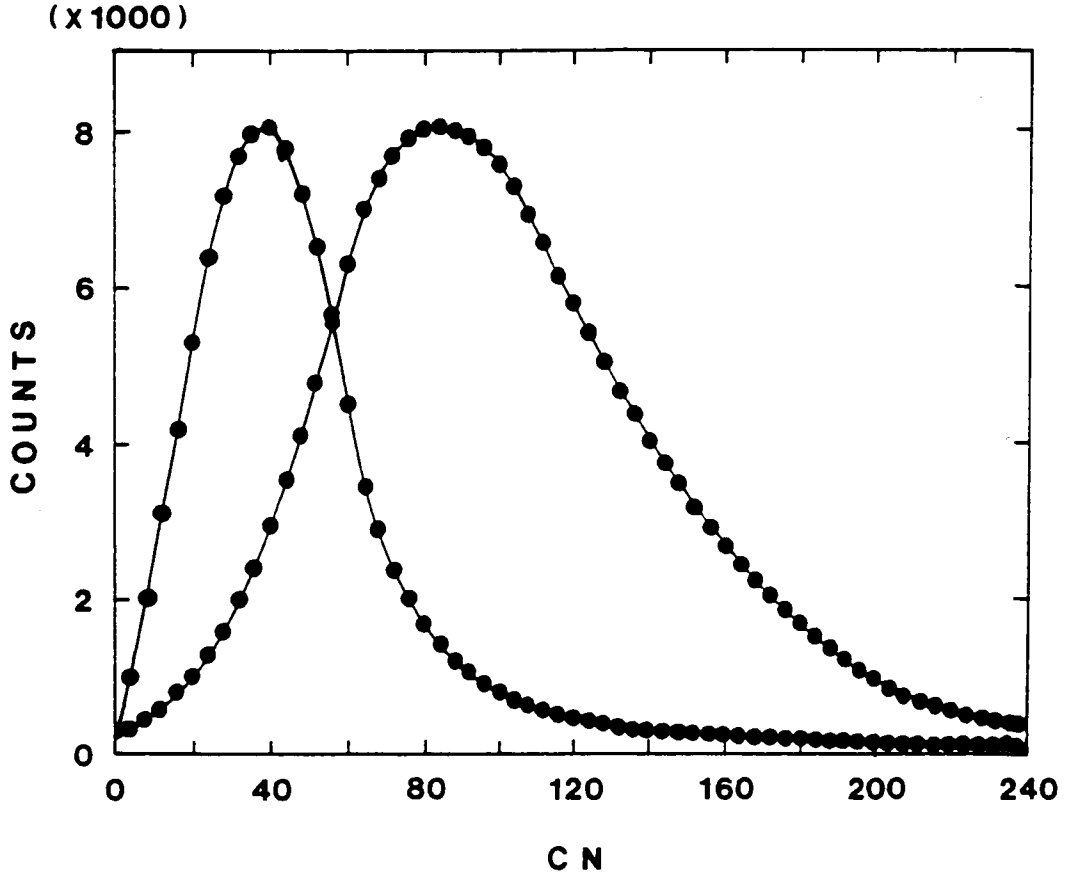



**Fig. 2-1.** *Fluorescence decay of 4-pyridoxic acid bound to RNase A (50 uM) in 0.1 M Hepes buffer (pH 7.0)*

Excitation wavelength was at 320 nm and the emission was filtered through a Corning glass filter (C-S-3-72). The deconvoluted biexponential decay function yields $\tau_1$ = 5.74 ns, $\alpha_1$ = 0.24, $\tau_2$ = 10.51 ns and $\alpha_2$ = 0.23. The average decay time, $\langle\tau\rangle$ = 8 ns, is included in Table 2-1.

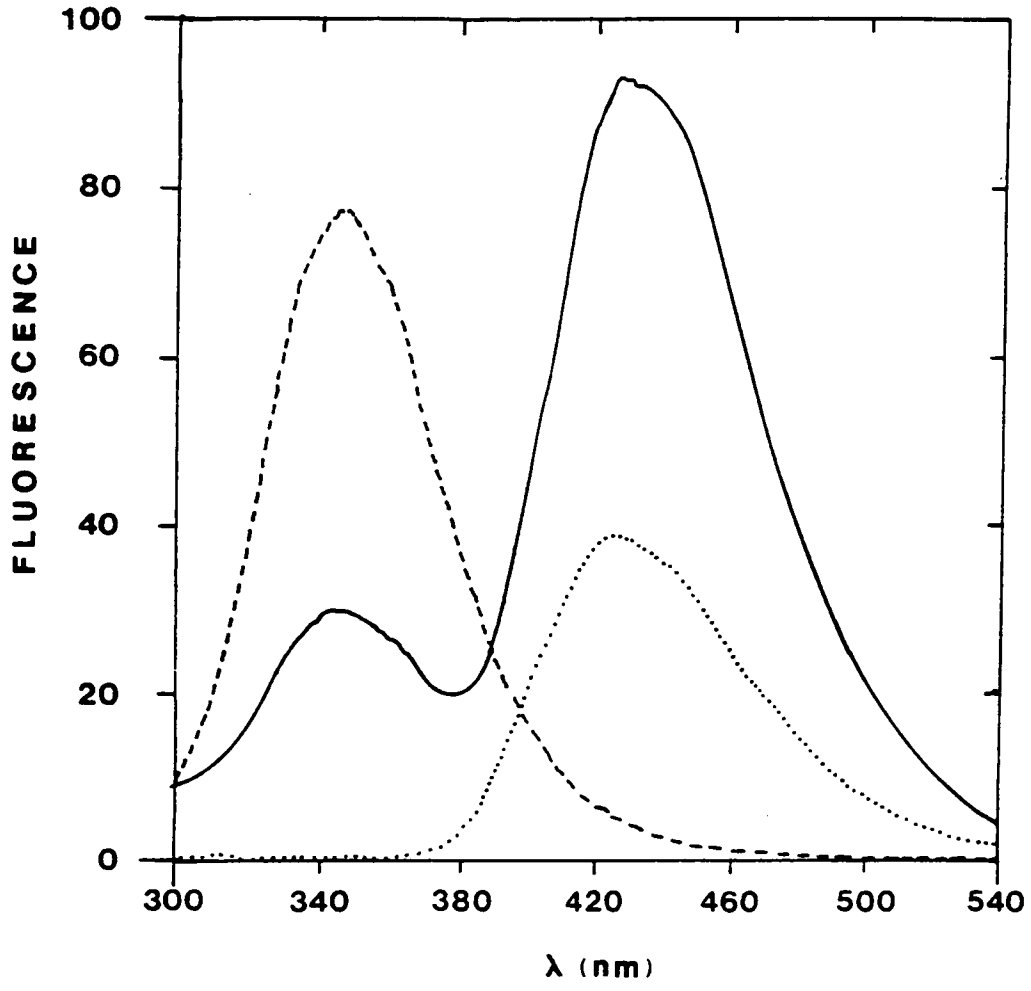



**Fig. 2-2.** *Intrinsic Energy Transfer ;*

*Donor : Tryptophan, Acceptor : Pyridoxic Acid*

Fluorescence emission spectra of pyridoxic acid (····), bovine serum albumin (---) and pyridoxic-BSA (–) by exicitation at 280 nm. The samples contained the same concentration of the acceptor and the donor.

Binding of Ligands to Modified Bovine Serum Albumin

The binding of two different ligands; *i.e.*, derivatized dipalmitoyl-phosphatidyl ethanolamine [N-(5-fluoresceinthiocarbamoyl)-1,2-dihexadecanoyl-sn-glycero-3-phospho ethanolamine; Fluorescein-DGPE)] and pyridoxal-5'-P to modified bovine serum albumin was monitored by two different methods; polarization of fluorescence and absorption spectroscopy, respectively.

In order to test the binding of the derivatized phospholipid to bovine serum albumin, the concentration of phospholipid was kept constant (3 $u$M) and the protein concentration varied over the range 0 - 50 $u$M in 0.1 M Hepes buffer (pH 7). After addition of the protein, the polarization of fluorescence was recorded at wavelengths longer than 500 nm upon excitation at 460 nm. As the protein concentration is increased, the observed polarization of fluorescence increases in the manner depicted in Fig. 2-3, until a limiting value of 0.38 is attained when the protein is saturated by fluorescein-DGPE. Judging from the results included in the figure, it appears that the incorporation of approximately 4 pyridoxic molecules / mol of protein does not influence the interaction between the derivative phospholipid and bovine serum albumin.

The binding of pyridoxal-5'-P to pyridoxic-BSA was monitored by differential absorption spectroscopy since the formation of a schiff's base between the carbonyl group of pyridoxal-5'-P and $\varepsilon$-$NH_2$ groups of the protein brings about an increase in the absorbance at 415 nm (Bempsey & Christensen, 1962). It has been shown that the carbonyl group of pyridoxal-5'-P reacts specifically with the $\varepsilon$-amino groups of lysine 220 and 223 of the primary sequence of the protein (Bohney *et al.*,1992). When pyridoxic-BSA (10 $u$M) was allowed to react with increasing concentrations of pyridoxal-5'-P (10-50 $u$M) at pH 7, and the spectra recorded after 20 minutes of incubation at 25°C, it was found that modified BSA failed to form a schiff's base with the extrinsic ligand. These results are interpreted to mean that activated pyridoxic acid has blocked the amino groups of the protein participating in the reaction with pyridoxal-5'-P.

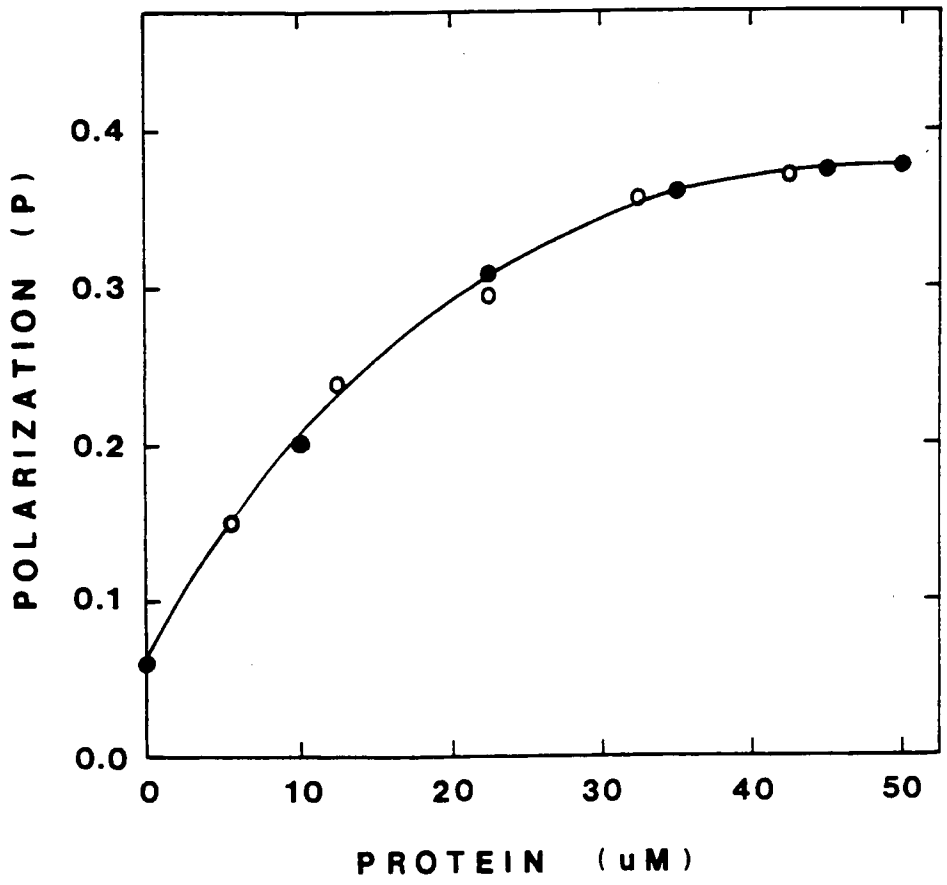



**Fig. 2-3.** *Titration of a fixed concentration of fluorescein-PE (3 uM) with varying concentration of bovine serum albumin.*

Changes in polarization of fluorescence (P) of fluorescein-PE obtained with increasing concentration of native BSA (o) and pyridoxic-BSA(•). Measurements were conducted in 0.1 M Hepes (pH 7.0) at 25 °C. Excitation wavelength was 460 nm and emission was selected by glass filters (Corning C-S-3-69).

Collisional Quenching

The degree of exposure of the fluorophore to collisional encounters with small molecules was derived from fluorescence intensity measurements under conditions of potassium iodide quenching. Dynamic fluorescence quenching can be described by Stern-Volmer equation ;

$$F_o/F = 1 + K_{sv}[Q] = 1 + k_q \tau_o [Q] \qquad (1)$$

where $F_o$ and F are the fluorescence intensities in the absence and presence of quencher [Q], $\tau_o$ is the fluorescence lifetime in the absence of quencher, $K_{sv}$, the Stern-Volmer quenching constant and $k_q$, the bimolecular quenching rate constant.

Fig. 2-4. shows Stern-Volmer plots for pyridoxic bound to the proteins bovine serum albumin and ribonuclease A together with free pyridoxic lactone in solution at pH 7. Using the observed values of fluorescence lifetimes, the bimolecular quenching rate constants included in Table 2-1 were obtained. A decrease in the bimolecular quenching rate constants for pyridoxic-BSA and pyridoxic-RNase A indicates that the fluorophore is partially shielded by the protein matrix from collisional encounters with iodide ions.

Polarization Spectra

The polarization of fluorescence spectra of the modified proteins was recorded upon excitation with varying wavelengths corresponding to the absorption transition of the probe. As expected, the polarization of fluorescence increases upon covalent attachment of pyridoxic acid to the protein, but the magnitude of the polarization values is influenced by the size of the globular protein bearing the fluorophore (Fig. 2-5). The pyridoxic-RNase complex is characterized by polarization of 0.1 at 320 nm excitation, whereas pyridoxic-BSA exhibits polarization of fluorescence values of 0.24 at the same excitation wavelength.

In marked contrast to the globular proteins, polylysine tagged with pyridoxic acid displays unusual low polarization of fluorescence values (P = 0.05) regardless of the

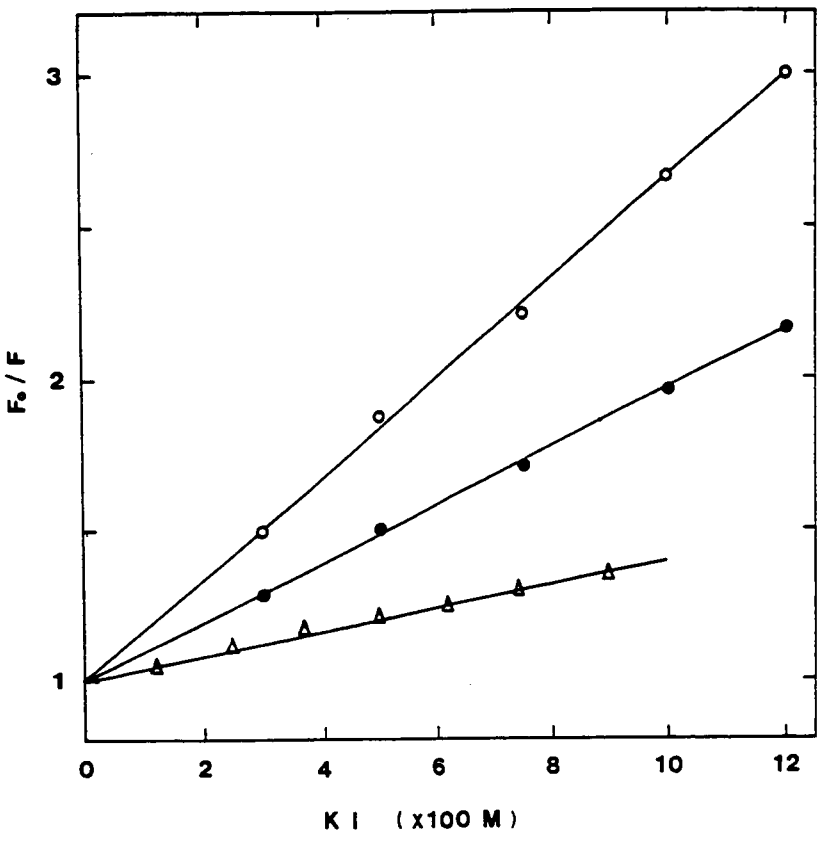



**Fig. 2-4.** *Stern-Volmer plots for iodide quenching of free pyridoxic lactone and pyridoxic bound to the proteins.*

The solid line represent the least square fit with parameter $K_{sv}$ = 16.7 $M^{-1}$, 9.6 $M^{-1}$ and 4.0 $M^{-1}$ for free pyridoxic lactone (o), pyridoxic-RNase (•) and pyridoxic-BSA (Δ), respectively. The bimolecular quenching constants ($k_q$) calculated from the eq. (1) are included in Table 2-1. Experiments performed at 25 °C in 0.1 M Hepes (pH 7.0) containing 0.2 M KCl and 0.1 mM sodium thiosulfate to maintain a constant ionic strength and to inhibit the formation of $I^{3-}$, respectively. Excitation wavelength was at 320 nm and the emission at 420 nm.

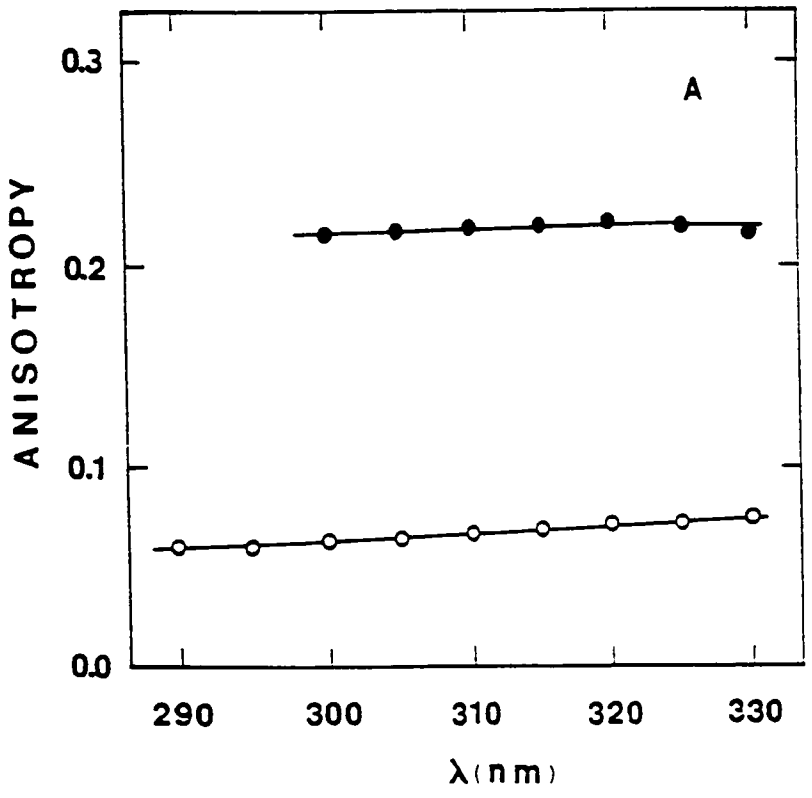



**Fig. 2-5.** *The excitation polarization spectra of pyridoxic-proteins*

The excitation polarization spectra of pyridoxic-RNase (o) and pyridoxic-BSA (•). The emission was filtered through Corning glass filters (C-S-3-72).

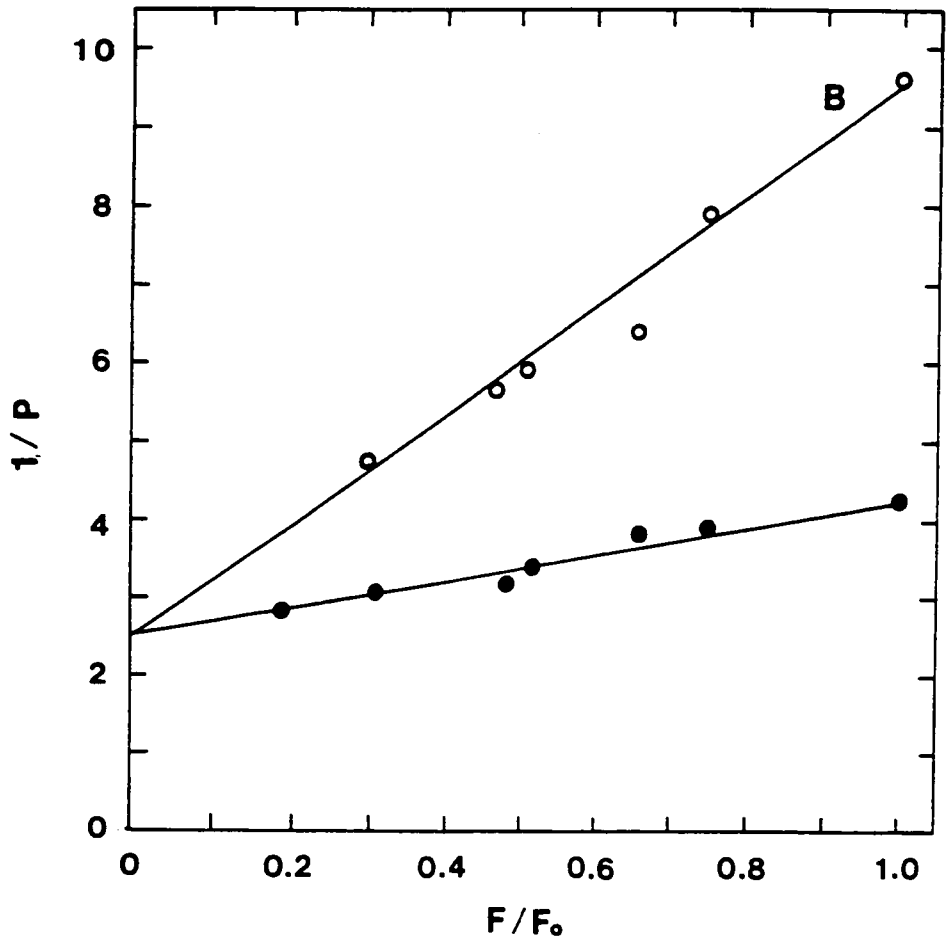



**Fig. 2-6.** *The apparent rotational correlation time for the modified proteins.*

Plots of polarization of fluorescence vs ratio of fluorescence intensities ($F/F_o$). The fluorescence intensities obtained by increasing concentration of quencher (KI). Open and filled circles represent pyridoxic-RNase and pyridoxic-BSA, respectively.

excitation wavelength and of the molecular mass of the polymer examined. At neutral pH, polylysine is a random coil and the flexibility of the side chains determine the magnitude of the observed polarization of fluorescence values. This behavior is characteristic of fluorescent probes that rotate independently of the whole macromolecule; *i.e.*, there is local motion of the probe at its site of attachment (Memming, 1963).

The observed polarization of fluorescence of proteins tagged with pyridoxic is influenced by the presence of collisional quencher's as indicated by equation (2).

$$\frac{1}{P} = \frac{1}{P_o} + \left(\frac{1}{P_o} - \frac{1}{3}\right) \cdot \frac{\tau_o}{\phi} \cdot \frac{F}{F_o} \qquad (2)$$

This equation is a modified form of Perrin's equation in which the fluorescence intensity (F) is proportional to the fluorescence life time ($\tau$) at certain quencher concentration [Q]. Plots of the reciprocal of the observed polarization of fluorescence against the ratio of fluorescence intensities ($F/F_0$) obtained in the presence of increasing concentrations of quencher (KI) follow a straight line as predicted by equation (2), Fig. 2-6.

Using this experimental approach, one obtains apparent rotational correlation times for RNase A and bovine serum albumin which are at least 2 times shorter than the values calculated for spherical hydration models of 14 kDa and 67 kDa molecular weight, respectively. Hence, it appears that the probe covalently attached to the proteins exhibits some degree of rotational mobility at its site of attachment (segmental motion). Perhaps, time dependent emission anisotropy measurements would separate the relative contribution of segmental and global motions to the apparent rotational correlation time determined by steady-state polarization of fluorescence.

Long Lived Luminescence

Luminescence studies of pyridoxal in the mixed solvent system ethanol : water, (95 : 5, v/v) at 77 °K have revealed the presence of a structureless emission band, which

decays with a lifetime of 1.2 seconds (Wampler & Churchich, 1969). This long lived emission; *i.e.*, phosphorescence, originates in the triplet state of pyridoxal detectable by electron spin resonance measurements (Wampler & Churchich, 1968). Like pyridoxal, pyridoxic acid shows phosphorescence in rigid glasses at liquid nitrogen temperature.

Several methods were tested in our attempts to detect phosphorescence emission of pyridoxic acid at room temperature in the absence of oxygen molecules. When $N_2$ gas was bubbled through the aqueous solution containing pyridoxic acid ($10^{-4}$M) and the phosphorescence recorded upon excitation at 320 nm, it was found that a structureless band centered at around 490 nm yields a decay time of 0.9 ms (Fig. 2-7, 2-8). Anaerobic conditions created by addition of glucose, glucose oxidase and catalase to solutions of pyridoxic acid in aqueous solutions of pH 7 (0.1 M Hepes buffer) yielded essentially the same results; *i.e.*, the emission band decays with a lifetime of 0.9 ms. When the same experiments were conducted in the presence of hexadecyl (trimethyl) ammonium bromide ($PamMe_3NBr$) (0.05 M) purged with $N_2$, the long lived luminescence was ten-fold enhanced ($Q_p = 10^{-3}$) when compared to solutions examined in the absence of micelles. Furthermore, the decay time was reduced from 0.9 ms to 0.02 ms.

Detergent molecules such as $PamMe_3NBr$ form micelles above a certain concentration range; *i.e.*, the critical micelle concentration. At low occupancy numbers, triplets may become virtually immune to deactivation by triplet-triplet annihilation and self quenching (Turbo *et al.*, 1980). Thus, triplet excited states formed in such micelles are protected from oxygen quenching and display enhanced phosphorescence (Turbo *et al.*, 1980).

Pyridoxic acid covalently bound to proteins and polylysine shows long lived emission at room temperature even when the protein concentration is around 20 $u$M (Fig. 2-7, 2-8). As soon as $O_2$ was incorporated into the sample, the long lived emission was not longer detectable. Table 2-1 lists the phosphorescence properties of the macromolecules tagged with pyridoxic acid.

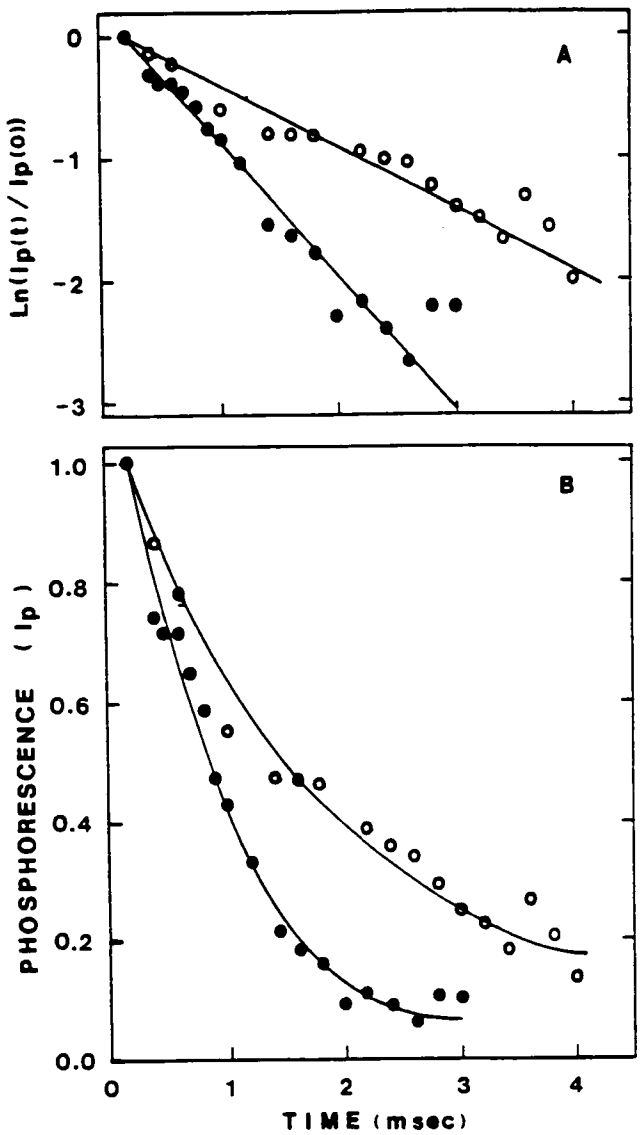



**Fig. 2-7.** *Phosphorescence properties of pyridoxic acid and pyridoxic-BSA.*

(A) Logarithmic plot of the relative phosphorescence intensity for pyridoxic (•) and pyridoxic-BSA(o). Regression analysis yields phosphorescence lifetime of 0.9 and 2.2 ms for pyridoxic and pyridoxic-BSA, respectively.

(B) Phosphorescence decay of pyridoxic acid (•) and pyridoxic-BSA (o). Excitation wavelength 320 nm, phosphorescence emission at 480 nm.

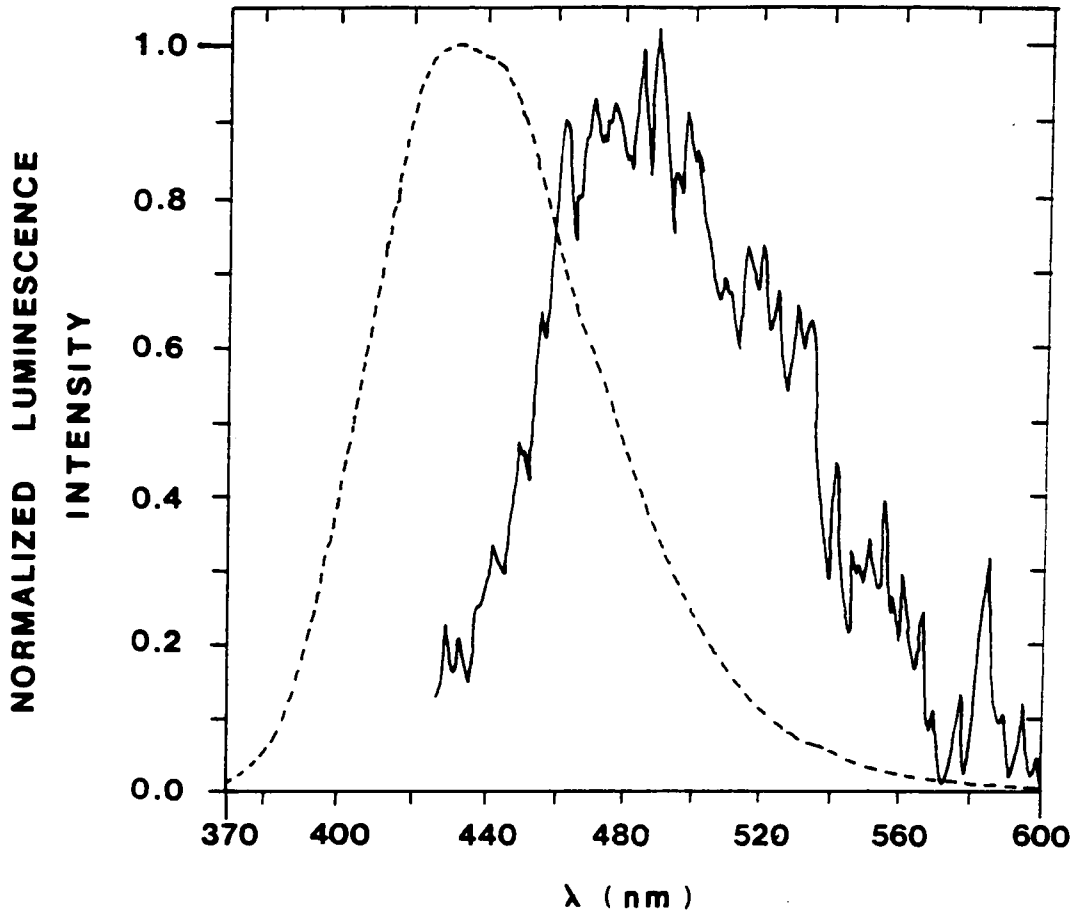



**Fig. 2-8.** *Luminescence spectroscopy of pyridoxic-BSA.*

Fluorescence emission spectrum (---) and phosphorescence emission spectrum (–) excitated at 320 nm. The phosphorescence spectrum was obtained by substrating the emission spectrum of fluorescence from the total luminescence, both as normalized spectrum. Hence, a red shifted spectrum (480 nm) is obtained for triplet excited state emission as compared to the singlet excited state (430 nm).

# DISCUSSION

The purpose of this study was to investigate the luminescence properties of pyridoxic acid covalently attached to proteins. The results of the labeling studies indicate that acylimidazole-pyridoxic reacts with proteins to yield stable amide linkages with lysyl residues.

Non-covalently binding of pyridoxic acid to bovine serum albumin has been previously examined by fluorescence techniques (Churchich, 1972). The recognition of pyridoxic acid by bovine serum albumin has some biological implications; 4-pyridoxic acid is the final product of the catabolism of vitamin $B_6$ (pyridoxal), and its binding to bovine serum albumin is probably important in the release and transport of this metabolite excreted in the urine. The concentration of bovine serum albumin in serum is very high (0.6 mM) (Peters, 1985) and its affinity for pyridoxic acid ($k_d$ = 6 $u$M) (Pineda, 1992) ensures non-covalent binding under physiological conditions.

The exact position in the primary sequence of the site of attachment of pyridoxic to bovine serum albumin has not been elucidated in the present work; but the finding that derivative bovine serum albumin failed to bind pyridoxal-5'-P which is known to react covalently with lysine residues 220 and 223 (Bohney *et al.*, 1992) of the primary sequence, lends strong support to the contention that both ligands share a common binding site.

Covalently binding of pyridoxic acid to the proteins examined causes an increase in the polarization of fluorescence without any significant change in the average fluorescence lifetime. In order to understand the changes in polarization of fluorescence of the labeled proteins, it is important to realize that the observed polarization is dependent on the rotational dynamics of the macromolecule bearing the chromophore. If the fluorescence lifetime is constant and if the extrinsic chromophore rotates in common

with the macromolecule, then one should observe a linear correlation between the reciprocal of the anisotropy (A = 2P / 3-P) and the reciprocal of the molecular weight of the macro-molecule as predicted by Perrin's equation for spherical bodies undergoing brownian motion.

For globular proteins like RNase A and bovine serum albumin the observed polarization of the probe is mainly influenced by the mass of the macromolecule undergoing rotational diffusion, whereas for random coils like polylysine, the flexibility of the side chains determines the magnitude of the polarization values. In view of these considerations it seems reasonable to suggest that pyridoxic acid is a suitable probe to detect fast rotational motions of macromolecules. Dynamic processes such as solvent relaxation and energy transfer, which often occur in the time scale of 0.1 - 10 ns, can also be detected by measuring the fluorescence properties; *i.e.*, quantum yield, band position and lifetime of the excited singlet state of pyridoxic covalently bound to proteins (Fig. 2-2).

One interesting aspect of this work is the finding that pyridoxic covalently attached to proteins exhibits long lived emission after excitation with a pulsed xenon source. On the basis of the results obtained in the absence of molecular oxygen at room temperature, the following arrangement of energy levels is proposed for pyridoxic acid; (1) a lowest singlet excited state corresponding to an energy level of 67 kcal/mol, (2) a metastable state; *i.e.*, triplet, characterized by an energy level of 57 kcal/mol.

The long lived emission characterized by a lifetime of 0.9 ms for free 4-pyridoxic acid might originate in the triplet state; *i.e.*, phosphorescence emission. Supporting this view is the observation that the heavy atom ($Br^-$) of the $PamMe_3NBr$ micelles and external quenchers such as $I^-$ enhance the intensity of the long lived emission with a concomitant decrease in the observed decay time. Alternatively, there is the possibility that the singlet excited state is populated by transitions from the triplet state to give delayed fluorescence. While this explanation seems attractive, it may be disputed on the

basis that the long lived emission spectra of either free or bound pyridoxic acid is red shifted when compared to the fluorescence spectra. Delayed fluorescence should exhibit the same emission spectrum as prompt fluorescence (McGlynn *et al.*, 1969).

Molecules in their triplet state have been used as probes of biologically relevant systems. Thus, tryptophan phosphorescence has been employed to monitor changes in the solution structure of proteins (Saviotti & Gally, 1974; Cioni *et al.*, 1993), whereas phosphorescence polarization of the extrinsic probe eosin linked to proteins has been used to measure slow motions of macromolecules attached to membranes (Garland & Moore, 1979; Cherry *et al.*, 1976). In the case of pyridoxic acid, two emission processes covering the time range $10^{-9}$ - $10^{-3}$ s can be easily detected at room temperature when the same probe is covalently linked to the protein. It is conceivable that fast and slow motions of proteins tagged with pyridoxic acid can be investigated by time dependent luminescence anisotropy techniques.

# PART II

# MOLECULAR CLONING :
# 4-AMINOBUTYRATE AMINOTRANSFERASE
# AND PYRIDOXAL KINASE

# CHAPTER 3

# ISOLATION AND SEQUENCE OF A cDNA ENCODING BRAIN 4-AMINOBUTYRATE AMINOTRANSFERASE

## INTRODUCTION

4-Aminobutyric acid is the major inhibitory neurotransmitter in mammalian brain. The concentration of 4-aminobutyric acid in brain is controlled by two pyridoxal-5'-P-dependent enzymes, *i.e.* glutamate decarboxylase and 4-aminobutyrate aminotransferase. The first enzyme catalyzes the synthesis of 4-aminobutyrate, whereas the second enzyme catalyzes the conversion of 4-aminobutyrate to succinic semialdehyde in a transamination reaction. The enzyme has been purified from a number of sources including rat (Bloch-Tardy *et al.*, 1971) and pig brain (Churchich & Moses, 1981) and from liver tissues (Buzenet *et al.*, 1978). In all cases the enzyme appears to be dimeric (~100 kDa) composed of identical subunits. Chemical analogs of 4-aminobutyrate, *i.e.* 4-aminohex-5-enoate, bind irreversibly to the aminotransferase, and it has been shown that some analogues are valuable drugs in the treatment of convulsive disorders (Lippert *et al.*, 1977). Further progress in the design of effective drugs for the treatment of convulsive disorders depends upon detailed information on the structure of the target enzyme. In order to further our understanding of the molecular structure and mechanism of the enzyme, we have isolated and sequenced a full-length cDNA encoding the pig brain enzyme.

# EXPERIMENTAL PROCEDURES

## Purification and Enzyme Assays

4-Aminobutyrate aminotransferase from pig brain was purified as described previously (Churchich & Moses, 1981). Enzymatic activity was determined by two methods. A coupled assay consisting of two purified enzymes, *i.e.* 4-aminobutyrate aminotransferase and succinic semialdehyde dehydrogenase, was used to study the catalytic conversion of 4-aminobutyrate to succinic semialdehyde. The progress of the reaction was monitored at 340 nm. A fluorometric method (Beeler & Churchich, 1978), based on fluorescence measurements of the condensation product of cyclohexane-1,3-dione with malonic semialdehyde, was applied to follow the conversion of $\beta$-alanine to malonic semialdehyde in the presence of $\alpha$-ketoglutarate at pH 8.

## Protein Sequence

Purified 4-aminoburyrate aminotransferase was reduced with tritiated $NaBH_4$, denatured with 5 M guanidinium HCl, and subjected to trypsin digestion as described by Kim & Churchich (1989). Individual peptides were isolated by chromatography on Vydac $C_{18}$ high performance liquid chromatography column, eluted with a 10-80% acetonitrile gradient in 20 mM trifluoroacetic acid. The sequence of two purified peptides was determined by automatic Edman degradation on a Beckman model 890 sequenator. One of the peptides, carrying labeled pyridoxyl, has the sequence Phe-Trp-Ala-His-Glu-His-Trp-Gly-Leu-Asp-Asp-Pro-Ala-Asp-Val-Met-Thr-Phe-Ser-Lys*-Lys. The other peptide has the sequence Ile-Asp-Ile-Pro-Ser-Phe-Asp-Trp-Pro-Ile-Ala-Pro-Phe-Pro-Arg.

## Preparation of Radiolabeled Probes

Two oligodeoxyribonucleotide probes designated Probe I and II, corresponding to the amino-terminal sequence (Kim *et al.*, 1984) and to the sequence of the pyridoxal-5'-P

binding site of pig brain 4-aminobutyrate aminotransferase, respectively, were used for oligonucleotide screening.

Probe I : 5' - GA(C/T)-GTN-CA(G/A)-TT(C/T)-GA(C/T)-TA(C/T)-GA(C/T)-GG - 3'
Asp Val Gln Phe Asp Tyr Asp Gly

Probe II : 5' - TT(C/T) - TGG - GCN - CA(C/T) - GA(G/A) - CA(T/C) - TGG - GG - 3'
Phe Trp Ala His Gly His Trp Gly

The oligonucleotides were synthesized using an Applied Biosystems, Inc. model 380 DNA synthesizer. They were labeled at their 5' ends using T4 polynucleotide kinase and [$\gamma$-$^{32}$P] ATP (Sambrook et al., 1989) and then purified with small Sephadex G-25 column ($1 \times 10^8$ cpm/$u$g).

For rescreening of a porcine brain cDNA library to obtain full length clones, a Kpn I / Eco RI restriction fragment of 495 bp was obtained from the isolated clone screened with the oligonucleotide probes and was labeled by nick translation (Benton & Davis, 1977). Unincorporated radioisotope was removed by spin column of Sephadex G-25. The specific activities of these probes were approximately $1 \times 10^9$ cpm/$u$g.

Screening of the cDNA Libraries

To screen the $\lambda$gt 11 porcine brain cDNA library with oligonucleotide probes, $2 \times 10^5$ bacteriophages were plated out and filter replicas were prepared using Biotrans Nylon membranes (ICN Biomedicals, Inc.). The filters were treated successively with 0.5 M NaOH, 1.5 M NaCl for 2min, 1.5 M NaCl, 0.5 M Tris-HCl (pH 8.0) for 5 min, and 3x SSC buffer (1x SSC (pH 7.0) contains 0.15 M NaCl plus 0.015 sodium citrate) briefly. The immobilization of the phage DNAs on the filters was performed by baking for 2-3 hrs at 80 °C. The filters were rinsed in 3x SSC containing 0.1 % SDS at 65 °C overnight with at least one change of solution and one rotation and then prehybridized in 6x SSC containing 2x Denhart's solution, 0.25 % SDS and 100 $u$g/ml denatured salmon sperm DNA at 42 °C for 5hrs with agitation. The hybridization was performed at 42 °C for overnight with the oligonucleotide probes denatured by heating at 100 °C. Approximately

2 to 4 ml of hybridization solution was used per 100 $cm^2$ of membrane. The filters were washed three times with 2x SSC containing 0.1 % SDS at r.t. and twice in the same solution at 40 °C successively. In order to select clones hybridizing more intensively with the probes from the positive clones, the hybridized filters were washed with the same solution but at higher temperatures (the temperature was raised at intervals of 10 °C).

Clones, which remained to be hybridized with the probe even when the filters were washed at near the melting temperature for the probe, were chosen for second, third screening, and further analysis of cDNA. Autoradiography for each washing step was performed using Kodak XAR films with an intensifying screen.

The protocol for rescreening with large nick-translated probes was similar to that shown in screening with oligonuclotide probes except following conditions for hybridization and washing. Filter bound DNA was prehybridized for 3-6 hrs. at 42 °C in 5x SSPE (1x SSPE (pH 7.4) equals 0.15M NaCl, 0.01M $NaH_2PO_4$ and 1mM EDTA), 0.1 % SDS, 5x Denhardt's solution, 50 % deionized formamide and 100 $u$g/ml of heat denatured salmon sperm DNA. Then the hybridization was performed for overnight at 42 °C with heat denatured probe ($1x10^6$ cpm/ml). The filter were washed twice each with 2x SSC containing 0.05 % SDS at room temperature for 1 hr. and with 1x SSC containing 0.1 % SDS at 68 °C for 1 hr. and dried on Whatmann paper prior to 8-24 hrs. of autoradiography.

Subcloning and Nucleotide Sequencing

Isolation of phage DNA and subcloning of cDNA inserts in the pBluescript phagemid vector (Stratagene, pBluescript II SK (-)) were conducted by the standard methods (Maniatis et.al., 1982). Phage DNA's were digested with a restriction enzyme Eco RI. The fragments were extracted from 1 % low melting point agarose gel and inserted into pBluescript phagemid vector with $T_4$-DNA ligase. The ligated products were used to transform *E. coli* DH5αF' cells. The color selection was made for identification of

recombinant clones within pBluescipt vector by plating on LB plates containing 50 $u$g/ml ampicillin, 80 $u$g/ml of fresh X-gal (5-bromo-4-chloro-3-indolyl-$\beta$-D-galactoside, a chromogenic substrate) and 20 mM IPTG (isopropylthio-$\beta$-D-galactoside, a gratuitous inducer of $\beta$-galactosidase). Colonies containing phagemids with inserts were remained white after incubation for 12-18 hrs. at 37 °C, whereas colonies without inserts were blue. The plasmid DNA was prepared by the alkaline lysis method followed by ribonuclease digestion and EtOH precipitation. Further purification was performed with pZ 523 spin column (5prime 3prime, Inc.) for DNA sequencing.

Sequence analyses were performed by the dideoxychain termination method (Sanger et.al., 1977) with $T_7$ DNA polymerase (Sequenase, USB Inc.). In general, double stranded DNA and synthetic oligonucleotides were used for the sequencing as a template and as primers, respectively. In some cases, single stranded DNA prepared with M13 helper phage and dGTP replaced by 5-deaza-dGTP to eliminate the compressions due to high GC content of the cDNA (Misuzawa et al., 1986).

Nucleotide and Deduced Amino acid Sequence Analyses

The nucleotide sequence and protein structure were analyzed with the aid of WARNER and GCG computer program, respectively. For DNA and protein homology search, GenBank data base were used.

Materials

Porcine cDNA library was obtained from Clontech laboratory, Inc. IPTG, X-gal and Salmon sperm DNA (type III sodium salt) was purchased from Sigma. The DNA was sheared by passing through a 17-gauge hypodermic needle for hybridization. Denhardt's reagent (Denhardt, 1966) made up as a 50x stock solution was filtered and stored at -20 °C. 50x Denhardt's reagent contains 5g of ficoll (Type 400, Pharmacia), 5g of polyvinylpynolidone and 5g of bovine serum albumin (pentex fraction V) in 500 ml $H_2O$.

[α-$^{32}$P]CTP was obtained from Du Pont - New England Nuclear. [γ-$^{32}$P]ATP and [α-$^{35}$S] ATP from ICN Biochemicals, Inc. and Amersham, respectively. Sephadex G-25 was purchased form Pharmacia.

## RESULTS AND DISCUSSION

Two oligonucleotide probes corresponding to the amino terminal sequence and to the sequence of the pyridoxal-5'-P binding site of pig brain 4-aminobutyrate aminotransferase, respectively, were used to screen a λgt 11 pig brain cDNA library. After the third screen, five candidates recognized the radioactive oligonucleotide probes. The cDNA of each positive clone was digested with *Eco* RI and electrophoresed on 0.8 % agarose gels. The cDNA inserts of the recombinant phages were ligated to *Eco* RI-digested pBluescript II SK(-) vector and amplified in *E. coli* DH5αF' cells. However, the sequence analysis showed that all isolated clones comprised only C-terminal portion of GABA-T gene. Thus, a fragment (495 bp) of the inserts digested with Kpn I/Eco RI was used for rescreening of the cDNA library to obtain full length clones. Using the fragment labeled by nick translation, four clones were isolated from $1.5x10^5$ bacteriophages of the library. The largest clone, containing the insert of 2.2 kilobases, was designated pBgabat-1 and used in further studies. The cDNA was fragmented by digestion with restriction enzymes, and each fragment was subcloned into its complementary site in the multiple cloning site of the pBluescript vector. A partial restriction map and the strategy for sequencing are shown in Fig. 3-1; the resulting sequence of the 4-aminobutyrate aminotransferase gene and the encoded protein are shown in Fig. 3-2.

The cDNA encoding for mature 4-aminoburyrate aminotransferase contains 1419 nucleotides. The size of the encoded polypeptide, 473 residues with $M_r$ = 53411, compares well with that reported earlier for the subunits of this enzyme. The deduced

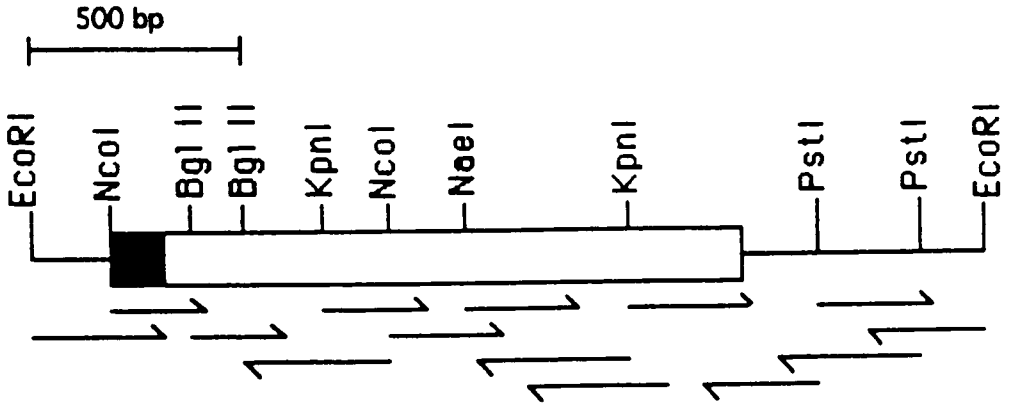



**Fig. 3-1.** *Nucleotide sequencing strategy and restriction map of the pig brain 4-aminobutyrate aminotransferase clones.*

The location of several restriction sites on the pig brain 4-aminobutyrate aminotrasferase cDNA are indicated. *Black and open* regions encode the aminoterminal precursor segment and mature protein, respectively. *bp*, base pairs.

AGC CGG GGT CTG GAG

GGC AGC AGC GTG TGA GGA GGG GCT CTG CTG GCG ACA GAG GAG ACC

1
ATG GCT TCC GTG TTG CTC ACC CGG AGG CTG GCC TGC AGC TTC CGC
**Met Ala Ser Val Leu Leu Thr Arg Arg Leu Ala Cys Ser Phe Arg**

46 *
CAC AAC CAC CGC CTG CTG GTG CCC GGA TGG AGA CAC ATC AGT CAG
**His Asn His Arg Leu Leu Val Pro Gly Trp Arg His** Ile Ser Gln

91
GCC GCA GCC AAA GTG GAC GTT GAA TTT GAT TAT GAC GGG CCT TTG
Ala Ala Ala Lys Val Asp Val Glu Phe Asp Tyr Asp Gly Pro Leu

136
ATG AAG ACG GAA GTC CCA GGG CCC AGA TCT CGG GAG TTA ATG AAA
Met Lys Thr Glu Val Pro Gly Pro Arg Ser Arg Glu Leu Met Lys

181
CAG CTG AAT ATA ATT CAG AAC GCA GAG GCT GTG CAC TTT TTC TGC
Gln Leu Asn Ile Ile Gln Asn Ala Glu Ala Val His Phe Phe Cys

226
AAT TAC GAA GAG AGC CGA GGC AAT TAC CTG GTG GAC GTG GAC GGC
Asn Tyr Glu Glu Ser Arg Gly Asn Tyr Leu Val Asp Val Asp Gly

271
AAC CGG ATG TTG GAT CTT TAC TCC CAG ATC TCC TCC ATC CCC ATA
Asn Arg Met Leu Asp Leu Tyr Ser Gln Ile Ser Ser Ile Pro Ile

315
GGT TAC AGC CAT CCT GCT CTC GTG AAA CTC GTC CAA CAG CCT CAG
Gly Tyr Ser His Pro Ala Leu Val Lys Leu Val Gln Gln Pro Gln

361
AAT GTG AGT ACA TTC ATT AAC AGA CCT GCC CTC GGA ATC CTT CCT
Asn Val Ser Thr Phe Ile Asn Arg Pro Ala Leu Gly Ile Leu Pro

406
CCG GAG AAC TTC GTG GAG AAG CTG CGG GAG TCC CTG CTC TCG GTG
Pro Glu Asn Phe Val Glu Lys Leu Arg Glu Ser Leu Leu Ser Val

451
GCT CCC AAA GGG ATG TCC CAG CTC ATC ACA ATG GCC TGC GGC TCC
Ala Pro Lys Gly Met Ser Gln Leu Ile Thr Met Ala Cys Gly Ser

496
AGC AAG GAA AGG GGC CAG TCG TGT TCC AAT GAA AAT GCG TTC AAG
Cys Ser Asn Glu Asn Ala Phe Lys Thr Ile Phe Met Trp Tyr Arg

541
ACC ATC TTT ATG TGG TAC CGG GCT TTC TCC AAA GAG GAG CTG GAG
Ser Lys Glu Arg Gly Gln Ser Ala Phe Ser Lys Glu Glu Leu Glu

586
ACG TGC ATG ATC AAC CAG GCC CCC GGG TGC CCC GAC TAC AGC ATC
Thr Cys Met Ile Asn Gln Ala Pro Gly Cys Pro Asp Tyr Ser Ile

631
CTC TCC TTC ATG GGT GCC TTC CAC GGG AGG ACC ATG GGT TGC TTA
Leu Ser Phe Met Gly Ala Phe His Gly Arg Thr Met Gly Cys Leu

676
GCG ACC ACG CAC TCC AAA GCC ATT CAC AAG ATC GAC ATC CCT TCC
Ala Thr Thr His Ser Lys Ala Ile His Lys Ile Asp Ile Pro Ser

721
TTC GAC TGG CCC ATC GCA CCA TTC CCG CGG CTG AAG TAT CCT CTG
Phe Asp Trp Pro Ile Ala Pro Phe Pro Arg Leu Lys Tyr Pro Leu

766
GAG GAG TTT GTG AAA GAG AAC CAA CAA GAA GAG GCC CGC TGT CTG
Glu Glu Phe Val Lys Glu Asn Gln Gln Glu Glu Ala Arg Cys Leu

811
GAA GAG GTG GAG GAC CTG ATT GTG AAA TAC CGG AAG AAG AAG AAG
Glu Glu Val Glu Asp Leu Ile Val Lys Tyr Arg Lys Lys Lys Lys

856
ACG GTG GCC GGC ATC ATC GTG GAG CCC ATC CAG TCT GAG GGC GGG
Thr Val Ala Gly Ile Ile Val Glu Pro Ile Gln Ser Glu Gly Gly

901
GAC AAC CAT GCG TCC GAC GAC TTC TTC CGG AAG CTG CGG GAC ATC
Asp Asn His Ala Ser Asp Asp Phe Phe Arg Lys Leu Arg Asp Ile

946
TCC AGG AAG CAC GGC TGT GCC TTC TTG GTG GAT GAG GTC CAG ACA
Ser Arg Lys His Gly Cys Ala Phe Leu Val Asp Glu Val Gln Thr

991
GGA GGA GGC TCC ACC GGC AAG TTC TGG GCC CAC GAG CAC TGG GGC
Gly Gly Gly Ser Thr Gly Lys Phe Trp Ala His Glu His Trp Gly

1036
TTG GAC GAC CCG GCC GAT GTG ATG ACC TTC AGC AAG AAG ATG ATG
Leu Asp Asp Pro Ala Asp Val Met Thr Phe Ser Lys Lys Met Met

1081
ACG GGG GGC TTC TTC CAC AAG GAG GAG TTC AGA CCC AAC GCT CCC
Thr Gly Gly Phe Phe His Lys Glu Glu Phe Arg Pro Asn Ala Pro

1126
TAC CGG ATC TTC AAC ACG TGG CTG GGG GAC CCT TCC AAG AAC CTG
Tyr Arg Ile Phe Asn Thr Trp Leu Gly Asp Pro Ser Lys Asn Leu

1171
CTG CTG GCC GAG GTC ATC AAC ATC ATC AAG CGG GAG GAC CTG CTG
Leu Leu Ala Glu Val Ile Asn Ile Ile Lys Arg Glu Asp Leu Leu

1216
AGC AAC GCA GCC CAC GCC GGG AAG GTC CTG CTC ACG GGG CTG CTG
Ser Asn Ala Ala His Ala Gly Lys Val Leu Leu Thr Gly Leu Leu

1261
GAC CTC CAG GCC CGG TAC CCC CAG TTC ATC AGC AGA GTG AGA GGA
Asp Leu Gln Ala Arg Tyr Pro Gln Phe Ile Ser Arg Val Arg Gly

1306
CGA GGC ACT TTT TGT TCC TTC GAC ACC CCA GAC GAA TCC ATA CGG
Arg Gly Thr Phe Cys Ser Phe Asp Thr Pro Asp Glu Ser Ile Arg

1351
AAT AAA CTC AAT TCC ATC GCC AGA AAC AAA GGT GTG ATG CTG GGG
Asn Lys Leu Asp Ser Ile Ala Arg Asn Lys Gly Val Met Leu Gly

1396
GGC TGT GGC GAC AAA TCC ATC CGC TTC CGT CCC ACT CTG GTC TTC
Gly Cys Gly Asp Lys Ser Ile Arg Phe Arg Pro Thr Leu Val Phe

1441
AGG GAT CAC CAC GCA CAC CTG TTC CTC AAT CTT TTC AGC GAC ATC
Arg Asp His His Ala His Leu Phe Leu Asn Ile Phe Ser Asp Ile

1486
TTA GCC GAC TTC AAG TAA AAA GCC ATT TCC TCC ACG CCC CGA GGA
Leu Ala Asp Phe Lys

AGC CCC GAT CTC AAC AAT CGT CCA AGT GAT

**Fig. 3-2.** *Nucleotide sequence of pig brain 4-aminobutyrate aminotransferase together with the deduced amino acid sequence.*

The nuceotide sequence surrounding the open reading frame of the cDNA is shown. The numbering is given beginning with the first base of the initiator methionine as base 1. The sequence of signal peptide and the first amino acid isoleusine for mature protein is indicated with bold letters and asterisk, respectively. Amino acid sequence derived from purified 4-aminobutyrate aminotransferase protein are indicated by *underlining.*

amino acid composition of the mature enzyme is shown in Table 3-1. The structural gene was identified by comparison of the encoded amino acid sequence with the previously determined partial $NH_2$-terminal sequence of this aminotransferase (Kim *et al.*, 1984). The deduced sequence of the mature enzyme coincides with the amino acid sequence of peptides previously determined by Edman degradation (Kim & Churchich, 1989). Thus, the sequence of three cysteinyl-containing peptides are easily identified in the coding region of the cDNA, whereas a peptide containing the cofactor binding site is located at nucleotide positions 1012-1074 of the gene. The cofactor pyridoxal-5'-P is covalently bound to lysyl residue 330 of the deduced amino acid sequence of the mature enzyme.A termination codon (TAA) is adjacent to the triplet coding for the carboxyl-terminal amino acid residue lysine. Neither a poly A tail nor a polyadenylation consensus sequence (AATAAA) was found in the sequences determined for the 3' untranslated region. As for the 5' untranslated region, a well defined ribosome binding site (GGAG) is separated from the first ATG codon by three bases.

The first methionine codon (ATG) is located at nucleotide positions 1-3 of the 5' end. Thus a sequence of 81 nucleotides coding for 27 amino acids is located in the open reading frame of the cDNA. It is suggested that the sequence of 27 amino acids, upstream of the amino-terminal isoleucine of the mature form of the enzyme pertains to the signal peptide of the precursor of 4-aminobutyrate aminotransferase. Consistency with this hypothesis is the finding that precursor 4-aminobutyrate aminotransferase was synthesized in a cell-free reticulocyte lysate as a protein of molecular mass larger than the mature form of the enzyme and displayed catalytic activity (Choi & Churchich, 1986). More recently, further evidence was shown in our laboratory that the recombinant precursor GABA-T expressed in E. coli was likely to be a dimer containing the cofactor pyridoxal 5'-phosphate and had catalytic activity (Park et al., 1993). These observations suggest that the binding of pyridoxal-5'-P occurs in the cytosol and the proteolytic removal of the signal peptide is relevant to the translocation into mitochondria. Like other

**TABLE 3-1**

*Amino Acid Composition of the mature 4-Aminobutyrate Aminotransferase*

| CODE | NUMBERS | % |
|---|---|---|
| Ala | 31 | 6.5 |
| Arg | 27 | 5.7 |
| Asn | 22 | 4.6 |
| Asp | 27 | 5.7 |
| Cys | 10 | 2.1 |
| Gln | 16 | 3.3 |
| Glu | 31 | 6.5 |
| Gly | 33 | 6.9 |
| His | 14 | 2.9 |
| Ile | 31 | 6.3 |
| Leu | 41 | 8.6 |
| Lys | 31 | 6.3 |
| Met | 13 | 2.7 |
| Phe | 30 | 6.3 |
| Pro | 25 | 5.2 |
| Ser | 33 | 6.9 |
| Thr | 18 | 3.8 |
| Trp | 5 | 1.0 |
| Tyr | 11 | 2.3 |
| Val | 24 | 5.0 |
| *TOTAL* | *473* | *100* |

Analysis done on the sequence in Fig. 3-2.

The molecular weight of the mature enzyme predicted is 53411

typical matrix-targeting signals (Douglas et al, 1986), the mitochondrial targeting signal of 27 amino acids contains basic amino acids every four to eight residues, a net positive charge, and exhibit the capacity to form amphipathic helix conformation.

The structure of 4-aminobutyrate aminotransferase summarized in Fig. 3-3 was analyzed with aid of GCG computer program. The hydropathy profile of 4-aminobutyrate aminotransferase indicated a rather even distribution of the hydrophobic and hydrophilic regions throughout the molecule as shown in Fig. 3-4. Therefore, no evidence for integral membrane domains in 4-aminobutyrate aminotransferase was found. Prediction of secondary structure (Garnier *et al.*, 1978) shown also in fig. 3-5A indicated the presence of $\alpha$–helices (33.0 %) and $\beta$-pleated sheets (22.2 %). In addition, Chou-Fasman prediction with Kyte-Doolittle hydropathy index is shown in fig. 3-5B. A computer calculation (program CHARGPRO) in Fig. 3-6 revealed that the isoelectric point for the mature protein is pH 7.25.

Searches of the protein data base revealed that the amino acid sequence of the brain enzyme bears significant homology (41.5 %) to the enzyme from yeast (Andre & Jauniaux, 1990) as shown Fig. 3-7. The sequences were aligned to give maximum identity with a minimal number of gaps (Devereux et al., 1984). Short regions of homology were noted between 4-aminobutyrate aminotransferase and aspartate aminotransferase but were considered of too low homology to be significant. It will be interesting to determine whether the structures of both enzymes are similar despite rather extensive divergences is their primary sequences. In this connection, it should be noted that preliminary X-ray analysis of the crystalline enzyme has been published (Housley *et al.*, 1990). The crystals diffract to 2.5 Å resolution, and there are two dimers per symmetric unit.

Specific DNA probes will be useful to study the level of transcription in various cell types as well as the size of mRNA of the enzyme. The mammalian enzymes involved in the GABA pathway such as glutamate dehydrogenase, succinic semialdehyde

dehydrogenase and GABA-T may also be expressed coordinatively for the regulation. It is also interesting to note that further work is underway in our laboratory is designated to isolate a human GABA-T clone by using a fragment of this isolated porcine cDNA as a probe. Moreover, the availability of recombinant 4-aminobutyrate aminotransferase will allow future studies using mutagenesis and chemical modification techniques to further define the action mechanism of this enzyme.

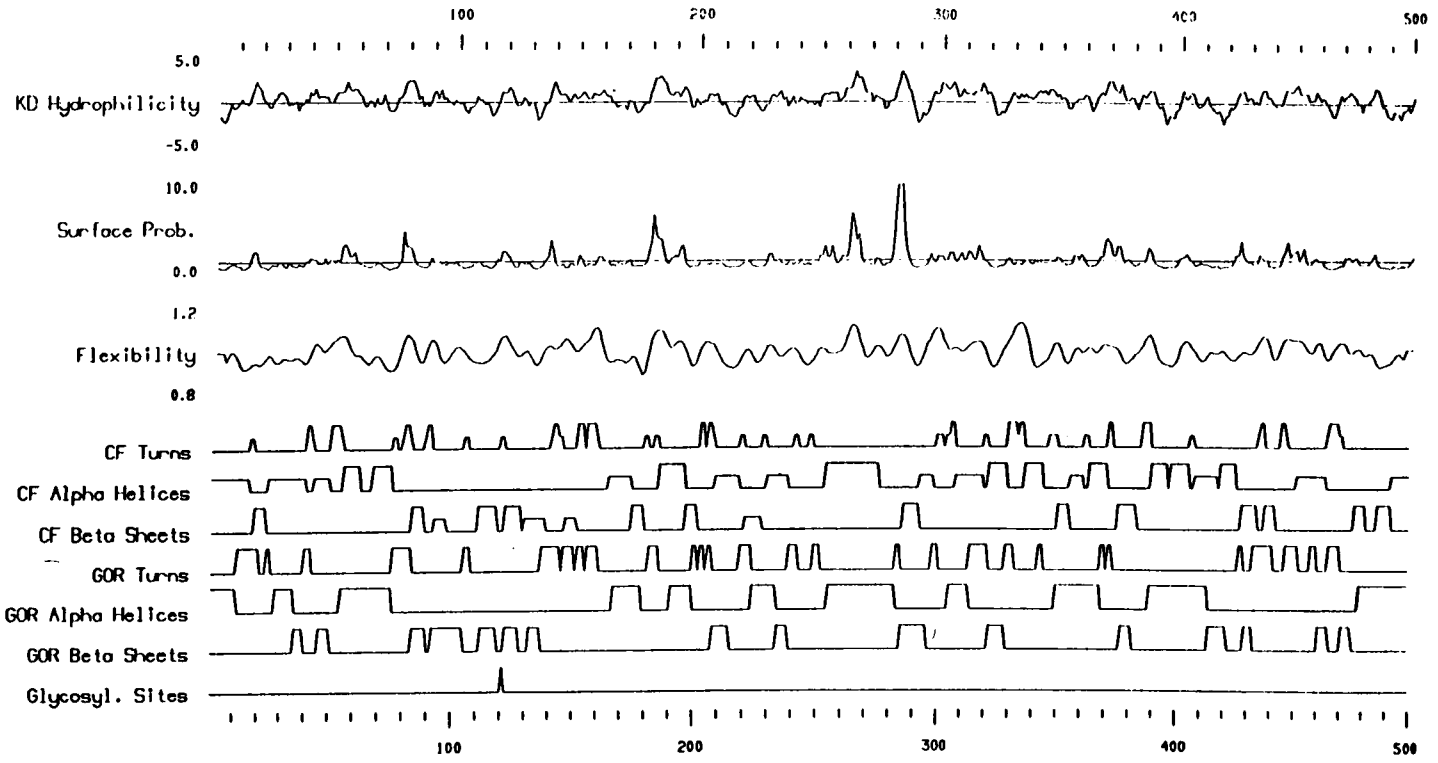



**Fig. 3-3.** *The predicted structures of precursor 4-aminobutyrate aminotransferase*

The hydropathy profile of the protein was determined by the method of Kyte-Doolittle (1982). In the predictions of secondary structure, CF and GOR represent Chou & Fasman, and Garnier-Osguthorpe-Robson, respectively.

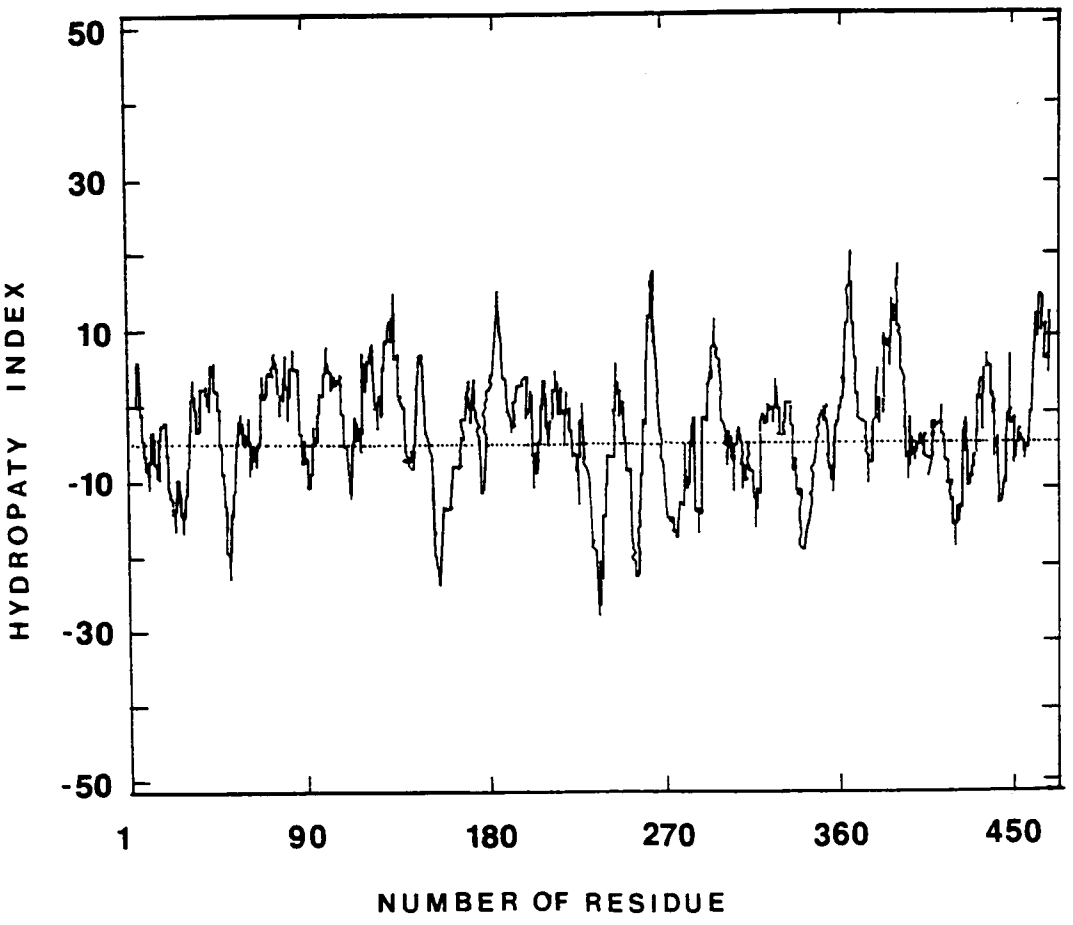
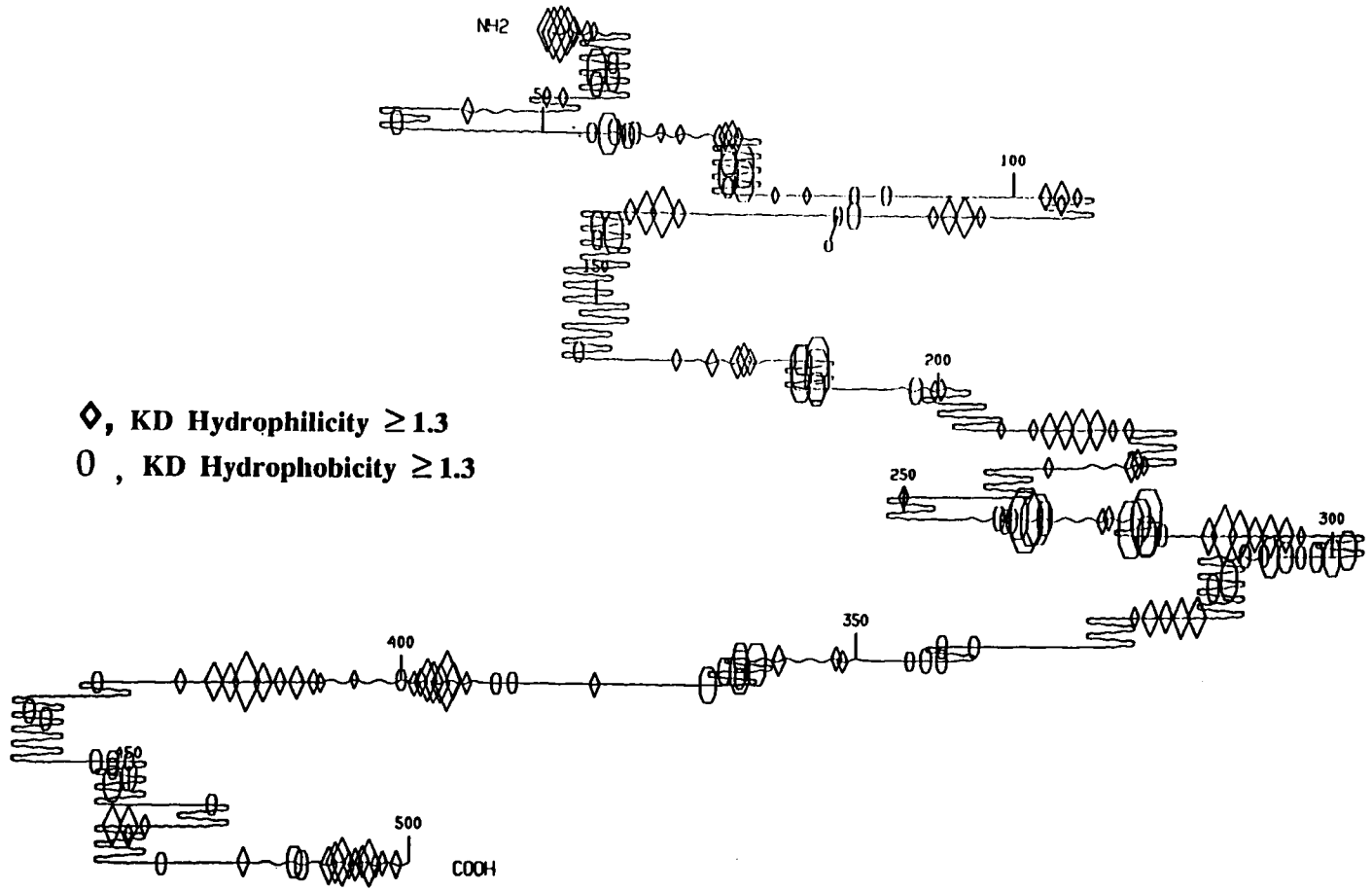



**Fig. 3-4.** *Hydropathy profile of mature 4-aminobutyrate aminotransferase*

Hydropathy index computated with a window size of 9 residues. The dotted horizontal line corresponds to a hydrophobicity value of -3.15. Amino acid numbers correspond to the deduced sequence of mature enzyme as shown in Fig. 3-7.

***Garnier-Osguthorpe-Robson Method***




◊, KD Hydrophilicity ≥ 1.3

0 , KD Hydrophobicity ≥ 1.3

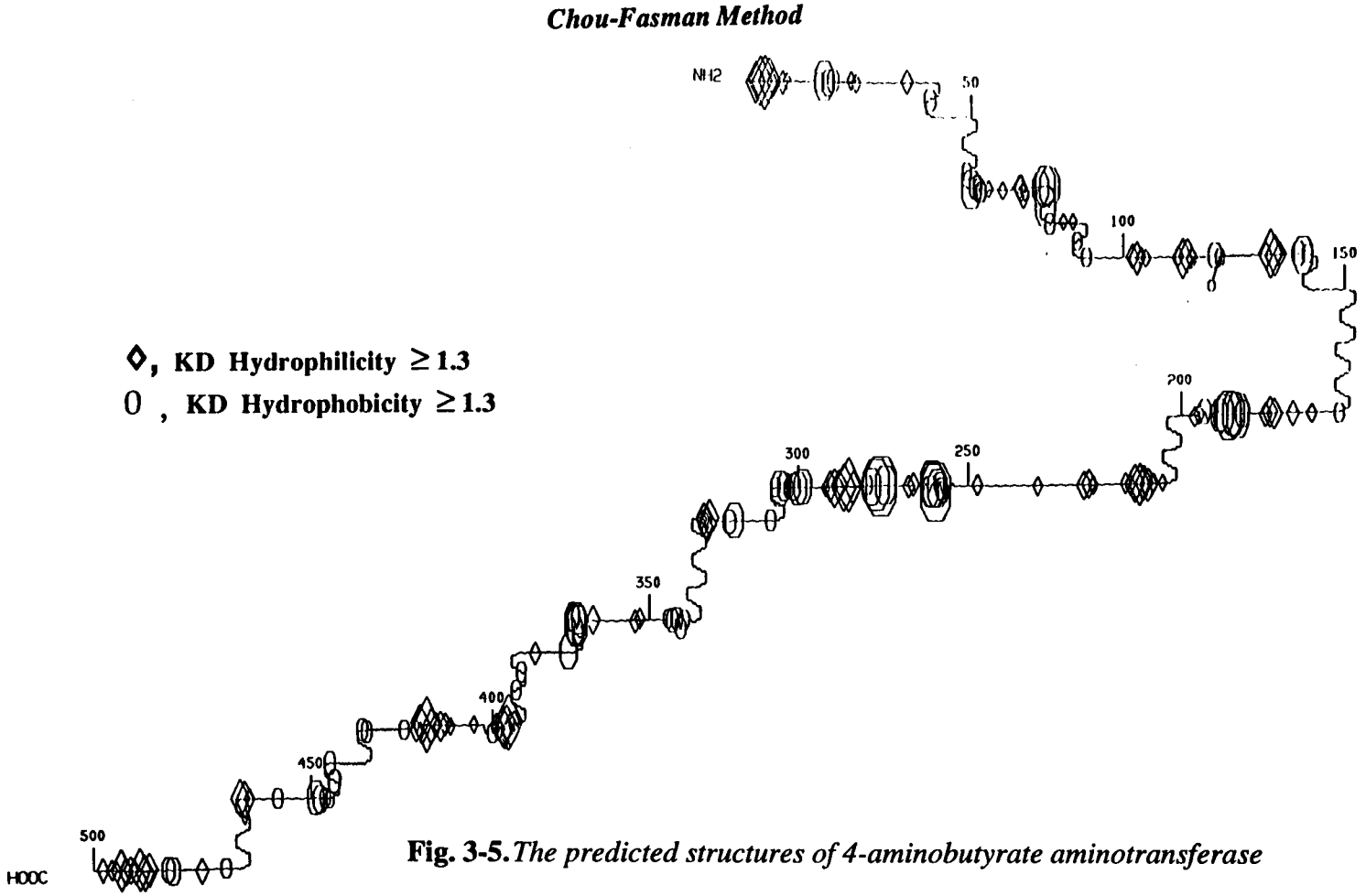



**Fig. 3-5.** *The predicted structures of 4-aminobutyrate aminotransferase*

The predictions were made with a computer program based on the Gornier-Osguthorpe-Robson (A) and the Chou-Fasman prediction (B). ◊, KD hydrophilicity ≥ 1.3. O, KD hydrophobicity ≥ 1.3.

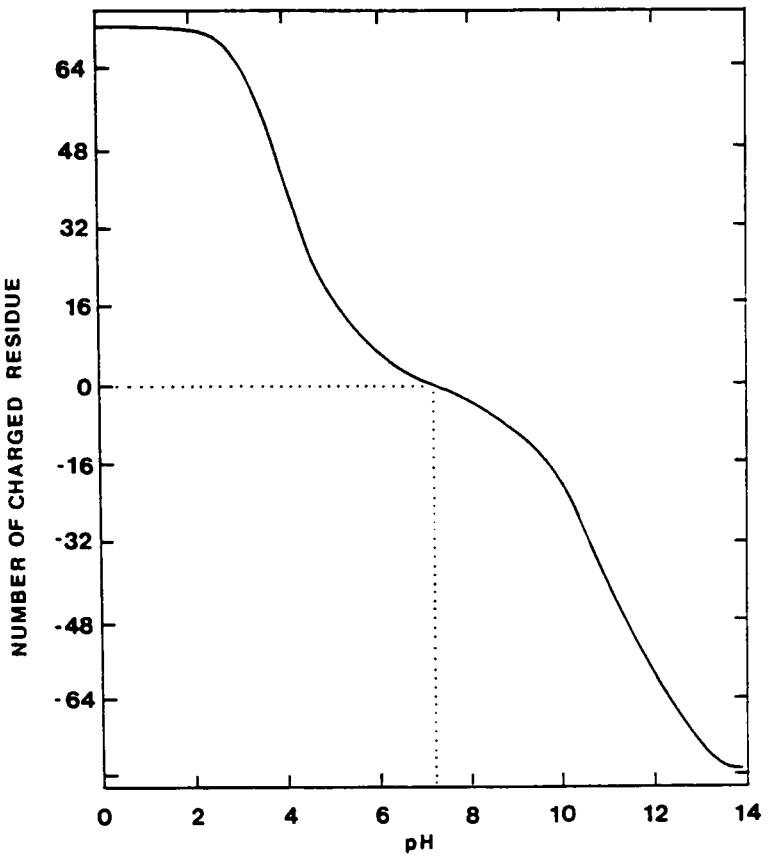



**Fig. 3-6.** *Isoelectric point of mature pig brain 4-aminobutyrate aminotransferase*

Isoelectric point of 7.25 was determined from the curve of the charge of the mature protein as a function of the pH.

```
          -27                                                    1
Pig    : MASVLLTRRLACSFRHNHRLLVPGWRHISQAAA
Yeast: MS ----------ICE----------------QYYPEEPT
          *           *

                                                25
Pig    :KVDVEFDYDGPLMKTE-VPGPRSRELMKQLNIIQN
Yeast:K--------------PTVKTESIPGPESQKQLKELGEVFD
         *               *   ***  ***  *     * *

                        50                                        75
Pig    :AEAVHFFCNYEESRGNYLVDVDGNRMLDLYSQISS
Yeast:TRPAYFLADYEKSLGNYITDVDGNTYLDLYAQISS
              *    ** * ***  ***** ****  ****

                                                100
Pig    :IPIGYSHPALVKLVQQPQNVSTFINRPALGILPPEN
Yeast:IALGYNNPALIKAAQSPEMIRALVDRPALGNFPSKD
        *  **   *** *   * *          *****  *

                              125
Pig    :FVEKLRESLLSVAPKGMSQLITMAC---GSCSNENA
Yeast:L--DKILKQILKSAPKG---QDHVWSGLSGADANELA
           *      *   ****   *          *    ** *

              150                                       175
Pig    :FKTIFMWYRSKERG--QSAFSKEELETCMINQAPGC
Yeast:FKAAFIYYRAKQRGYDADFSEKENLSVMDNDAPGA
        **   *   ** * **      **   *     * * ***

                                          200
Pig    :PDYSILSFMGAFHGRTNGCLATTHSKAIHKIDIPSF
Yeast:PHLAVLSFKRAFHGRLFASGSTTCSKPIHKLDFPAF
        *      ***   ***** *        ** ** *** * * *

                        225                                       250
Pig    :DWPIAPFPRLKYPLEEFVKENQQEEARCLEEVEDLI
Yeast:HWPHAEYPSYQYPLDENSDANRKEDDRCLAIVEELI
          * * *   *    *** *      *   *   ***  ** **

                                                275
Pig    :VKYRKKKKTVAGIIVEPIQSEGGDNHASDDFFRKL
Yeast:-----KTWSIPVAALIIEPIQSEGGDNHASKYFLQKL
                   **   * ********** ****  *   **

                              300
Pig    :RDISRKHGCAFLVDEVQTGGGSTGKFWAHEHWGLD
Yeast:RDITLKYNVVYIIDEVQTGVGATGKLWCHEYADIQ
        ***   *          ******  * ***  *  **

             325                                       350
Pig    :DPADVMTFSKKMMTGG--FFHKEEFRPNAPYRIFNT
Yeast:PPVDLVTFSKKFQSAGYFFHDPKFIPNKPYRQFNT
          *  *   *****      *  ***    * **  *** ***
```

```
                                                  375
Pig    :WLGDPSKNLLLAEVINIIKREDLLSNAAHAGKVLLT
Yeast:WCGEPARMIIAGAIGQEISDKKLTEQCSRVGDYLFK
       *  *  *                 *      *          *    *

                        400
Pig    :GLLDLQARYPQ--FIS--RVRGRGTFCSFDTPDESIRN
Yeast:KLEGLQKKYPENFQNLRGKGRGTFIAWDLPTGEKRD
       *    **   **   *    *   *****    *  *      *

     425                                          450
Pig    :KLNSIARNKGVMLGGCGDKSIRFRPTLVFRDHHAH
Yeast:LLLKKLKLNGCNVGGCAVHAVRLRPSLTFEEKHAD
       *           *     ***        *  **  *  *    **

                                473
Pig    :LFLDIFSDILADFK
Yeast:IFIEALAKSVNEL--
       *
```

**Fig. 3-7.** *Comparison of the primary structure in porcine and yeast GABA-T.*

The identical residues are indicated by asterisk and the numbering is given beginning with the first amino acid isoleucine as amino acid 1 for the mature porcine protien.

# CHAPTER 4

# MOLECULAR CLONING OF BRAIN PYRIDOXAL KINASE

## INTRODUCTION

Pyridoxal 5'-phosphate is the cofactor required by numerous enzymes that catalyze transamination and carboxylation reactions. The formation of pyridoxal phosphate from ATP, pyridoxal and a divalent cation ($Zn^{2+}$) is catalyzed by pyridoxal kinase (McCormick *et. al.,* 1961) which is an essential enzyme of the metabolism of vitamin $B_6$. This enzyme has been purified from various tissues and characterized in several laboratories (Kwok & Churchich, 1979; Kerry *et. al.,* 1986). The enzyme isolated from sheep brain is a monomer of 40 kDa that tends to associate into dimers of 80 kDa. Limited chymotryptic digestion resulted in the release of two fragment of 24 kDa and 16 kDa with concomitant loss of catalytic activity (Dominici & Churchich, 1989). Further studies have suggested that both the nucleotide and the pyridoxal binding site are located in the 24-kDa structural domain (Scholz *et al.*, 1990). These results suggest that the flexible polypeptide chain, i.e. hinge bending domain, connecting two structual domains is involved in opening and closing the cleft betweeen the two fragments for the catalysis. Recent NMR studies have suggested that the phosphorylation of pyridoxal can occur via direct transfer of the phosphoryl group between ATP and pyridoxal without formation of phosphorylated enzyme intermediate (Willem *et al.*, 1991). More recently, Pineda & Churchich (1993) showed from equilibrium denaturation studies that the nucleotide-binding domain is more stable than the pyridoxal-binding domain of the enzyme.

Despite these structural and functional studies, the X-ray structure of pyridoxal kinase is still not avilable although the enzyme has been crystalized recently (Arnone *et al.*, 1989). Moreover, no sequence is available so far for the enzyme. In order to have further understanding of the enzyme, several putative cDNA clones for the pyridoxal kinase have been isolated and sequenced from porcine brain as well as sheep liver cDNA library.

## EXPERIMENTAL PROCEDURES

### Enzyme Purification

The method used in the purification of sheep brain pyridoxal kinase is based on methods originally applied to the purification of this enzyme from pig (Kwok & Churchich,1979). The enzyme was purified from sheep brain tissues by Kwok, F (Kerry et al., 1986). Brifly, the purification procedure includes a step of affinity chromatography on pyridoxal-agarose and elution with 10 mM pyridoxal. Using 20 kg of sheep brain as starting material, 35 mg of pure enzyme was obtained.

### Amino Acid Analysis

The proteins (5 nmol) were hydrolyzed for 24 hr in 6N HCl, containing 0.1 % thioglycolic acid at 110 $^{o}$C in vacuum. Aminoacid derivatized with phenylisothiocyanate (PhNCS) were identified and quantified by HPLC (Waters) PICO-TAG using a NOVA-PAK $C_{18}$ column run at room temperature with a flow rate of 1ml/min.

### Synthesis of Oligodeoxynucleotide Probes

For screening of cDNA library containing the pyridoxal gene, the following two oligonucleotide probes were designed on the basis of amino acid sequence data of sheep liver pyridoxal kinase.

Probe I : 5' - A(GA)N-AC(AG)-TG(AG)-CAC-ATN-GC - 3'

Probe II : 5'- GA(CT)-TA(CT)-(CT)TN-ATG-GCN-(CT)T - 3'

The Probe I and II are corresponding to the complementary sequence of Ala-Met-Cys-His-Val-Leu and to the sequence of the Asp-Tyr-Leu-Met-Ala-Leu, respectively, obtained from the trypsin digested peptide sequence.

The oligonucleotides synthesized were radiolabeled at their 5' ends using $T_4$ polynucleotide kinase and [$\gamma$-$^{32}$P]ATP (7000Ci/mmol; Manniatis at al. 1982) and then purified with spin column of Sephadex G-25. The specific activities were approximately $1x10^8$ cpm/$\mu$g.

Screening of the cDNA Libraries

To screen the lgt 11 porcine brain or sheep liver cDNA libraries (Clontech, Inc) with oligonucleotide probes, $2.5x10^5$ bacteriophages were plated out, and filter replicas were prepared using Biotrans Nyron membranes (ICN Biomedicals, Inc.). The filters , in general, were hybridized, rinsed and analyzed with same ways as described in "Experimental Procedures" of GABA-T cloning.

Subcloning and Nucleotide Sequencing

Isolation of phage DNA and subcloning of cDNA inserts generated by *Eco* RI digestion in the pBluescript phagemid vector (Stratagene, pBluescript II SK (-)) were conducted by the standard methods (Maniatis et.al., 1982). The plasmids were amplified in *E. coli* DH5$\alpha$F' cells. Sequence analyses were performed by the dideoxychain termination method (Sanger et.al., 1977) with $T_7$ DNA polymerase (Sequenase, USB Inc.). Double stranded DNA and synthetic oligonucleotides were used as a template and as primers, respectively, for the sequencing. When needed to eliminate occasional compression artifacts, dITP was used in place of dGTP in the sequencing reactions.

Nucleotide and Deduced Amino acid Sequence Analyses

The Nucleotide sequence of the pyridoxal kinase was analyzed with the aid of a computer program, WARNER. For DNA and protein homology search, GenBank data base were used.

## RESULTS AND DISCUSSION

Initial screening of porcine brain and sheep liver cDNA libraries with the two oligonucleotide probes corresponding to the peptide sequences obtained by trypsin digestion yielded 7 positive clones from $2.5x10^5$ clones of each library screened. These clones were subjected to further screening, and 4 and 5 candidate clones which strongly recognized the radioactive oligonucleotide probes were obtained from porcine and sheep library, respectively. The autoradiographic replica of one of the candidates is shown in Fig. 4-1. The size of each insert was estimated to be1.2 - 1.5 kilobase pair from *Eco* RI digestion. The insert fragment was then subcloned into the pBluescript SK (-) phagemid, and transformed to the DH5$\alpha$F' competent cell for the double strand DNA sequencing.

One of amino acid sequences used to design the oligonucleotide probe (Probe II) was found in an open reading frame of the insert. The other sequence (for Probe I), however, was found in 5' -untranslated region. The actual nucleotide sequences are a little different from the those of probes used for the screening as shown in Fig. 4-2. Surprisingly, there was no difference between the sequence from porcine and that from sheep library.

Although it was clear all isolated clones with various sizes had been derived from the same gene since their sequences completely overlapped with one another, none of the putative clones contained a full length of open reading frame for the enzyme. It is well known that the pyridoxal kinase, purified from sheep brain tissues, is a 40 kDa (Kerry, *et*

*al.*, 1986). In order to cover a complete sequence (330-350 aminoacid residues), the nucleotide size for the open reading frame must be at least 1kb. Although the full-length cDNA was not obtained, the composition of predicted sequence is rather similar to that determined experimentally (data not shown). Searches of the sequence data base revealed that the nucleotide and the amino acid sequence deduced from the isolated cDNA clones does not bear any significant homology to other mammalian enzyme.

From these results, one cannot conclude that the clones isolated are the genes for pyridoxal kinase, although the amino acid composition predicted from the cDNA sequence is comparable with that of the enzyme. In order to be able to deduce more information about these clones, more amino acid sequence of pyridoxal kinase will be needed for matching them. If portions of the genes isolated are found to match the sequence of amino acid, the longer fragments of the isolated gene could be used for rescreening to obtain a full length cDNA.

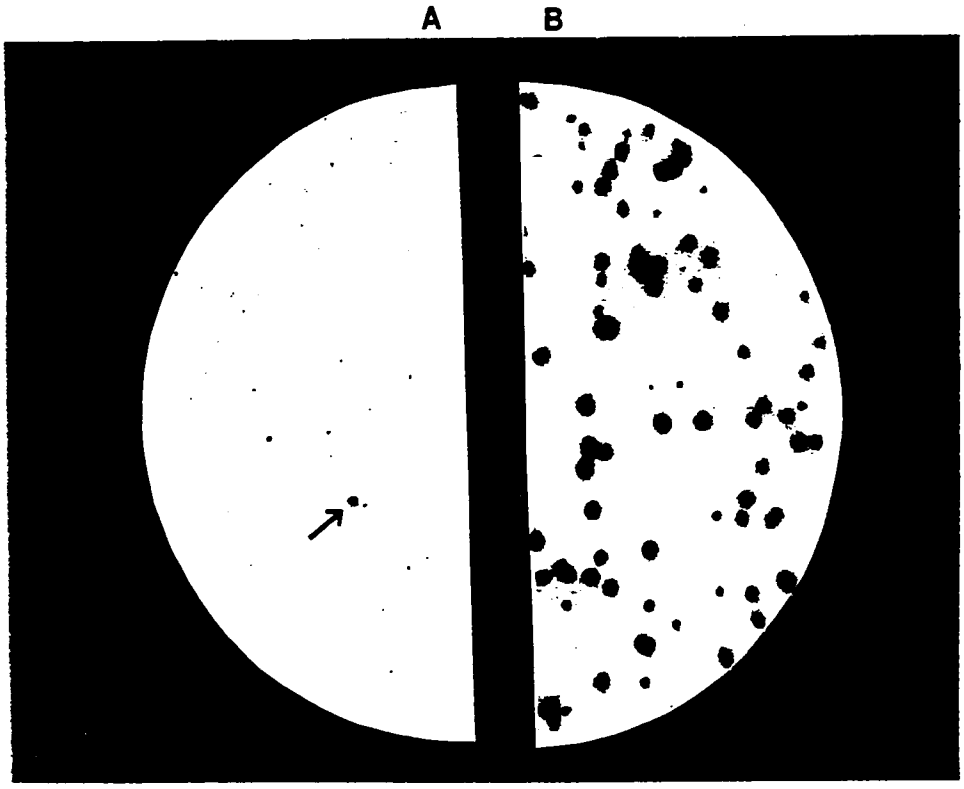



**Fig. 4-1.** *A λgt 11 cDNA colon containing sheep liver pyridoxal kinase.*

λgt 11 phages of sheep liver cDNA library were plated on E. coli Y1090 cells, and screened with radioactive oligonucleotide probes. (A) A positive clone indicated with an arrow was recognized by the probes in a primary screening. The candidate clone was isolated and amplified for further screening shown in (B).

```
1 TTATGAAGTGTCACGTTCTTTGGCTGCCGCCGAAGGGCCATTTTAGTCGT 50
      *  S  V  T  F  F  G  C  R  R  R  A  I  L  V  V
60 CGATGCTTCACAAGGCGTGGAAGCACAAACGTTAGCCAATGTTTATTTGG 100
    D  A  S  Q  G  V  D  A  Q  T  L  A  N  V  Y  L
101 CTTTAGATAACGGCTTGGAAATTTTGCCAGTCATTAATAAAATTGATCTC 150
   A  L  D  N  G  L  E  I  L  P  V  I  N  K  I  D  L
151 CCAGCCGCTGATCCGGAAAAGGTCCGTGCTGAGATTGAAGATGTGATTGG 200
    P  A  A  D  P  G  K  V  R  A  E  I  E  D  V  I  G
201 GATTGATGCTTCAGAAGCCGTGTTAGCTTCTGCTAAAAAAGGGATTGGCA 250
    I  D  A  S  E  A  V  L  A  S  A  K  K  G  I  G
251 TTCCAGAATTGCTGGAAAAGATTGTTACGGATTTGCCTGCACCAACAGGG 300
   I  P  E  L  L  E  K  I  V  T  D  L  P  A  P  T  G
301 GATTTGGAAGCACCATTACGGGCCTTGATTTTTGACTCGGCTTATGACCC 350
    D  L  E  A  P  L  R  A  L  I  F  D  S  A  Y  D  P
                        *
351 ATACCGTGGTGTTGTGGTCAACATGCGTGTGCGTGAAGGGATTGTGAAGC 400
    Y  R  G  V  V  V  N  M  R  V  R  E  G  I  V  K    9

401 CTGGTGATAAAATTCGCATGATGAACACTGGTGCTGAGTATGAAGTCAAT 450
10 P  G  D  K  I  R  M  M  N  T  G  A  G  Y  E  V  N   26

451 GAAGTCGGCGTGATGTCACCAAATGCGCAAAAGCGTGATTATTTGATGGC 500
27  E  V  G  V  M  S  P  N  A  Q  K  R  D  Y  L  M  A  43

501 TGGTGATGTTGGTTATTTGACGGCTTCTATCAAAGACATTCATACGACGC 550
44   G  D  V  G  Y  L  T  A  S  I  K  D  I  H  T  T    59

551 ATTCTGGTGATACGATTACCAGTGTGGCGAATCCTGCCTCTGAACCGTTG 600
60 H  S  G  D  T  I  T  S  V  A  N  P  A  S  E  P  L   76

601 GCCGGTTATAAGCCGATGACACCAATGGTTTATTCAGGACTATACCCATC 650
77  A  G  Y  K  P  M  T  P  M  V  Y  S  G  L  Y  P  S  93

651 TGATAATGCCAAATATACGGATTTGCGTGATGCGTTAGAAAAGTTGCAGT 700
94   D  N  A  K  Y  T  D  L  R  D  A  L  E  K  L  Q   109
```

```
701 TAAACGACGCGGCGTTGACATTTGAACCGGAAACATCACAAGCCTTGGGC 750
110 L  N  D  A  A  L  T  F  E  P  E  T  S  Q  A  L  G 126

751 TTTGGTTACCGTGTTGGGTTCCTCGGGTTGTTGCACATGGATGTGATTCA 800
127  F  G  Y  R  V  G  F  L  G  L  L  H  M  D  V  I  Q 143

801 GGAACGACTAGAACGTGAATTTGATTTGGATCTTGTGACGACAGCACTTT 850
144    E  R  L  E  R  E  F  N  L  D  L  V  T  T  A  L  159

851 CGGTTACTTACCGTGTGTTGACCAACGATGGCGAAGAATTAGAAATTGAA 900
160 S  V  T  Y  R  V  L  T  N  D  G  E  E  L  E  I  E 176

901 AACCCATCAGAAATGCCGGAGCCATCTAAGGTGAAGGCGATTGAAGAACC 950
177  N  P  S  E  M  P  E  P  S  K  V  K  A  I  E  E  P 193

951 CTATGTTGATGCTCAAATTATGGTGCCCAACGAATATGTTGGCGCCGTCA 1000
194   Y  V  D  A  Q  I  M  V  P  N  E  Y  V  G  A  V   209

1001 TGGAATTGGCACAACGTAAGCGTGGTGAATTTGAAACGATGGAGTATTTG 1050
210 M  E  L  A  Q  R  K  R  G  E  F  E  T  M  E  Y  L 226

1051 GATGCGTCACGTGTTAATGTGAAATATAAGATGCCGTTGTCAGAAATTAT 1100
227  D  A  S  R  V  N  V  K  Y  K  M  P  L  S  E  I  I 243

1101 TTTCAATTTCTTTGACAAATTAAAATCAAGTACACGTGGCTATGCCTCGC 1150
244   F  N  F  F  D  K  L  K  S  S  T  R  G  Y  A  S   259

1151 TAGATTATGAAATTTCTGGTTATCGTCCATCTGACTTGGTCAAGATT    1197
260 L  D  Y  E  I  S  G  Y  R  P  S  D  L  V  K  I     275
```

**Fig. 4-2**. *Nucleotide and deduced amino acid sequence of a clone for pyridoxal kinase.* Two sequences similar to those used for the probes are underlined.

# PART III

## *myo*-INOSITOL MONOPHOSPHATASE :

## REVERSIBLE UNFOLDING STUDIES AND

## INTERACTION WITH 70 kDa HEAT SHOCK COGNATE PROTEIN

# CHAPTER 5

## REVERSIBLE UNFOLDING OF *myo*-INOSITOL MONOPHOSPHATASE

### INTRODUCTION

*myo*-inositol monophosphatase catalyses dephosphorylation of *myo*-inositol-1-phosphate to *myo*-inositol, which is needed for resynthesis of phosphatidyl inositol (Hallcher & Sherman, 1980; Berridge, 1984; Majerus *et al.*, 1988). A soluble enzyme catalyzing $Li^+$ sensitive hydrolysis of *myo*-inositol-1-phosphate has been completely purified from rat brain (Takimoto *et al.*, 1985) and shown to be similar to the enzyme isolated from bovine brain (Gee *et al.*, 1988; Meek *et al.*, 1988). *myo*-inositol monophosphatase also catalyses the hydrolysis of a few other compounds, including 2'-AMP and β-glycerophosphate (Takimoto *et al.*, 1985), which is a convenient and inexpensive substrate for routine studies. The complete c-DNA and deduced primary amino acid sequence of the bovine brain enzyme has been reported (Diehl *et al.*, 1990). The protein is a homodimer comprising 29 kDa subunits.

Relatively little information has been reported on the structure and identification of the active site of this enzyme. In an attempt to increase our understanding of the structure and stability of *myo*-inositol monophosphatase, we have decided to investigate the hydrodynamic properties of the protein and the stability of the dimeric structure in the presence of the denaturing agent guanidine hydrochloride (Gu·HCl). *myo*-inositol monophosphatase treated with various concentration of Gu·HCl recovers full catalytic activity upon dilution of the denaturant. The analysis of the reversible denaturation curves obtained in Gu·HCl by different physical techniques, *i.e.*, circular dichroism and emission

anisotropy, is useful for the investigation of the physical properties of the folded and unfolded states thereby giving insight into the mechanism of folding.

## EXPERIMENTAL PROCEDURES

### Purification of *myo*-Inositol Monophosphatase

The procedure developed by Meek *et.al.* (1988) for the purification of bovine brain *myo*-inositol monophosphatase was used in the isolation of the enzyme from pig brain. The major purification steps are (a) removal of interfering proteins by heat denaturation at 75°C for 1 hour, (b) separation by DEAE-Sephacel at pH 6, (c) adsorption of remaining impurities on a Procion Red sepharose column and (d) chromatography on mono-Q anion exchange column (FPLC).

Starting with 6.4 kg of fresh pig brain, 16mg of pure inositol monophosphatase were obtained. Purity of the enzyme was tested on SDS polyacrylamide gel electrophoresis using 12.5% polyacrylamide gels. The specific activity of the purified enzyme was 8 *u*mole/min·mg with D,L-β-inositol-1-P and 4 *u*mole/min·mg with D,L-β-glycerophosphate at pH 7.4, 25°C. Protein concentration was determined by the method of Bradford (1976).

### Enzymatic Assays

Inorganic phosphate was assayed with malachite green as described by Lanzetta *et al.* (1979). Enzymatic assays were performed at pH 7.5 in 10 mM Tris·HCl buffer containing β-glycerophosphate (2mM), $MgCl_2$ (20mM) and NaCl (0.1M). Stock solution of the enzyme (1mg/ml) in the absence and presence of Gu·HCl at pH 7.5 were kept at 4°C for 3 hours. Then they were brought to 25°C and kept at that temperature for 1 hour prior to the enzymatic assays. The time courses of product formation were obtained by

diluting the enzyme (10 *u*l) into the reaction mixture (1ml) at 25°C. At various time intervals, 2, 3, 5, 7 and 10 minutes, 50 *u*l aliquots were removed, mixed with 1ml of malachite green / ammonium molybdate, incubated for 30 minutes at 25 °C, and read at 660 nm in the spectrophotometer.

Labeling of Inositol Monophosphatase

The enzyme at a concentration of 4 mg/ml was allowed to react with1,5-IAEDANS in 0.1 M HEPES buffer (pH 7.5) at 4°C. The reaction was allowed to proceed for 6 hrs. Excess free reagent was removed by dialysis against 50 mM Tris·HCl (pH 7.5), followed by gel filtration through a sephadex G-25 column equilibrated with the Tris·HCl buffer.

The degree of labeling of the enzyme (1.2 mole/dimer) was determined spectrophotometrically using an extinction coefficient of 6.2 $x10^3$ $M^{-1}cm^{-1}$ at 340 nm. The labeled enzyme was fully active.

Sample Preparation for CD and Fluorescence Spectroscopy

The samples used for fluorescence and circular dichroism measurements were dissolved in 10 mM Tris·HCl buffer (pH 7.5). For denaturation experiments, each sample was prepared by diluting 0.2 ml of protein stock solutions to 2ml with Gu·HCl containing buffer. All measurements were carried out at 25°C after 1 hour incubation in the presence of Gu·HCl. Control experiments indicated that this time of incubation was sufficient to achieve equilibrium.

Circular Dichroism Spectra

These were recorded on a Jasco (J-40A) spectropolarimeter using a 1 cm cell. The spectrometer was routinely calibrated with the asymmetric compound (+)-10-camphorsulfonic acid (CSA). Protein concentrations ranged from 0.035 to 0.1 mg/ml and

spectral data were acquired over the range 250-200 nm. Ellipticity [θ], expressed in terms of mean residue ellipticity was calculated with the aid of the equation (1);

$$[\theta] = \theta \cdot MRW / 10 \cdot l \cdot C \qquad (1)$$

where MRW is the mean residue weight, l, path length (1cm) and C, concentration in g/ml.

Fluorescence Spectroscopy

Emission anisotropy measurements were carried out on an SLM polarization apparatus. Illumination provided by a xenon lamp (150 watts) was passed through a grating monochromator. Emission light was filtered through glass filters (C.S-3-72, Corning). Emission anisotropy values were recorded on a fluorimeter built in our laboratory. It consists of two Baush Lomb monochromators, a xenon light source and a detector (EMI photomultiplier). The absorbance of the samples at the excitation wavelengths was around 0.1 (1 cm cuvette). Emission decay measurements were made using the monophotonic technique in an Ortec nanosecond spectrometer. A free running flash lamp was pulsed at 10 kHz and the excitation set at 340 nm. The emission was filtered through a glass filter (C.S-3-72, Corning). Fluorescence decay kinetics of the chromophore 1,5-I-AEDANS bound to the enzyme was deconvoluted and analyzed by a nonlinear least square method (Greenvald & Steinberg, 1974). Adequacy of the exponential decay fitting was judged by inspection of weighted residual plots and the reduced $\chi^2$. Anisotropy decay measurements were performed by recording the fluorescence decay curves of the polarized components $I_{/}(t)$ and $I_{\perp}(t)$, parallel and perpendicular to the plane of the incident polarized light. Polaroid HNB sheet polarizers were used for excitation and emission. Anisotropy decay analysis was performed using the method of the "sum and difference" (Wahl, 1979; O'Connor & Phillips, 1984). The exponential function $d(t) = I_{/}(t) - I_{\perp}(t)$ was deconvoluted and analyzed by a nonlinear least-square method. The deconvoluted curves were fitted to the equation ;

$$I_{//}(t) - I_{\perp}(t) = 3 A_o \cdot e^{-t/\phi r} \cdot e^{-t/\tau_f} \quad (2)$$

where $\tau_f$ is the fluorescence decay time of the function $s(t) = I_{//}(t) + 2I_{\perp}(t)$ measured in a separate experiment. $\phi_r$ is the rotational correlation time.

High Performance Liquid Chromatography

HPLC was performed on a LKB instrument and a TSK 3000 SW column (600 x 7.5 mm) was used in the experiments. The mobile phase was 10 mM Tris.HCl buffer (pH 7.5) containing various concentrations of Gu.HCl for the analysis of dissociation of the enzyme at 25 $^{o}$C. The flow rate was 0.2 ml/min and the column was calibrated with standards of known molecular markers ; carbonic anhydrase (30 kDa), Ovalbumin (45 kDa), bovine serum albumin (67 kDa) and phosphorylase *b* (94 kDa). The elution profiles were monitored by recording absorbance at 230 nm. Experiments using different Gu.HCl concentrations were repeated twice.

## RESULTS

Stability of the Dimeric Structure

The purification procedure developed by Meek *et al.* (1988) is perhaps exceptional with regard to its simplicity ; the enzyme *myo*-inositol monophosphatase from pig brain could be purified in less than 3 days. Purified inositol monophosphatase exhibits a molecular weight of 58,000 when examined by the technique of gel filtration chromatography and it dissociates into monomers under drastic denaturation conditions as revealed by SDS polyacryamide gel electrophoresis (Gee *et al.*, 1988).

The rotational correlation time of the catalytic species in solution at neutral pH was determined by time dependent emission anisotropy measurements. The monophosphatase was tagged with the fluorescent probe AEDANS, which reacts with SH

groups of the protein without impairing the catalytic parameters. The fluorescence of the probe covalently attached to the protein decays in a bi-exponential manner with fluorescence lifetimes of 19.1 and 10 ns, respectively (Table 5-1). The average lifetime $<\tau>$ = 18 ns reflects the predominant contribution of the longest lifetime component characterized by an amplitude of $\alpha$ = 0.8. The rotational correlation times of the monophosphatase were determined at two different protein concentrations, *i.e.*, 1 and 6 *u*M, using time-correlated single photon counting (O'Connor & Phillips, 1984).

A typical set of experimental results is shown in Fig. 5-1, together with the statistical evaluation of the fitting procedure. A very fast initial decay of the emission anisotropy function from $A_D$ = 0.3 to 0.25 takes place in less than 3 ns, suggesting subnanosecond flexibility of the macromolecule. Thereafter, the emission anisotropy function decays in a monoexponential manner over the time interval 4-24 ns.

The experimental data collected over the time interval 4-24 ns could be fitted to the function ;

$$A(t) = A_D \cdot e^{-t/\phi_r} \qquad (3)$$

where $\phi_r$ is the rotational correlation time of the whole macromolecule tagged with the probe. The rotational correlation time determined for inositol monophosphatase in solution ($\phi_r$ = 30 ns) remains constant when the protein concentration is decreased to 1 *u*M (Table 5-1). Hence, no dissociation of the dimeric protein was detected at concentrations of 1 *u*M. The overall correlation time for a spherical hydrated macromolecule (h= 0.2 g $H_2O$ / g protein) of 58 kDa, calculated with the aid of equation (4) and (5) ;

$$\phi_o = V\eta / k \cdot T \qquad (4)$$

$$V = (\upsilon + h) \cdot M / N_D \qquad (5)$$

yields a rotational correlation time of $\phi_o$ = 22 ns for a partial specific volume of $\upsilon$ = 0.73. The experimental value of 30 ns indicates either a deviation from spherical symmetry (prolate ellipsoid of axial ratio = 3) or an increase in the degree of hydration of

**TABLE 5-1**

*Rotational Correlation Times of AEDANS-monophosphatase*

| Sample | Fluorescence Lifetime (ns) | | | | Rotational Correlation Time (ns) |
|---|---|---|---|---|---|
| | $\tau_1$ | $\tau_2$ | $\alpha_1$ | $\alpha_2$ | |
| Enzyme (6 *u*M) | 19.1 | 10.0 | 0.80 | 0.20 | 30 |
| *Enzyme (4 *u*M) | 19.2 | 10.0 | 0.81 | 0.19 | 29 |
| Enzyme (1 *u*M) | 19.0 | 9.9 | 0.79 | 0.21 | 29.6 |

*Sample treated with 1M Gu·HCl and dialyzed against 50 mM Tris·HCl buffer (pH 7.5)

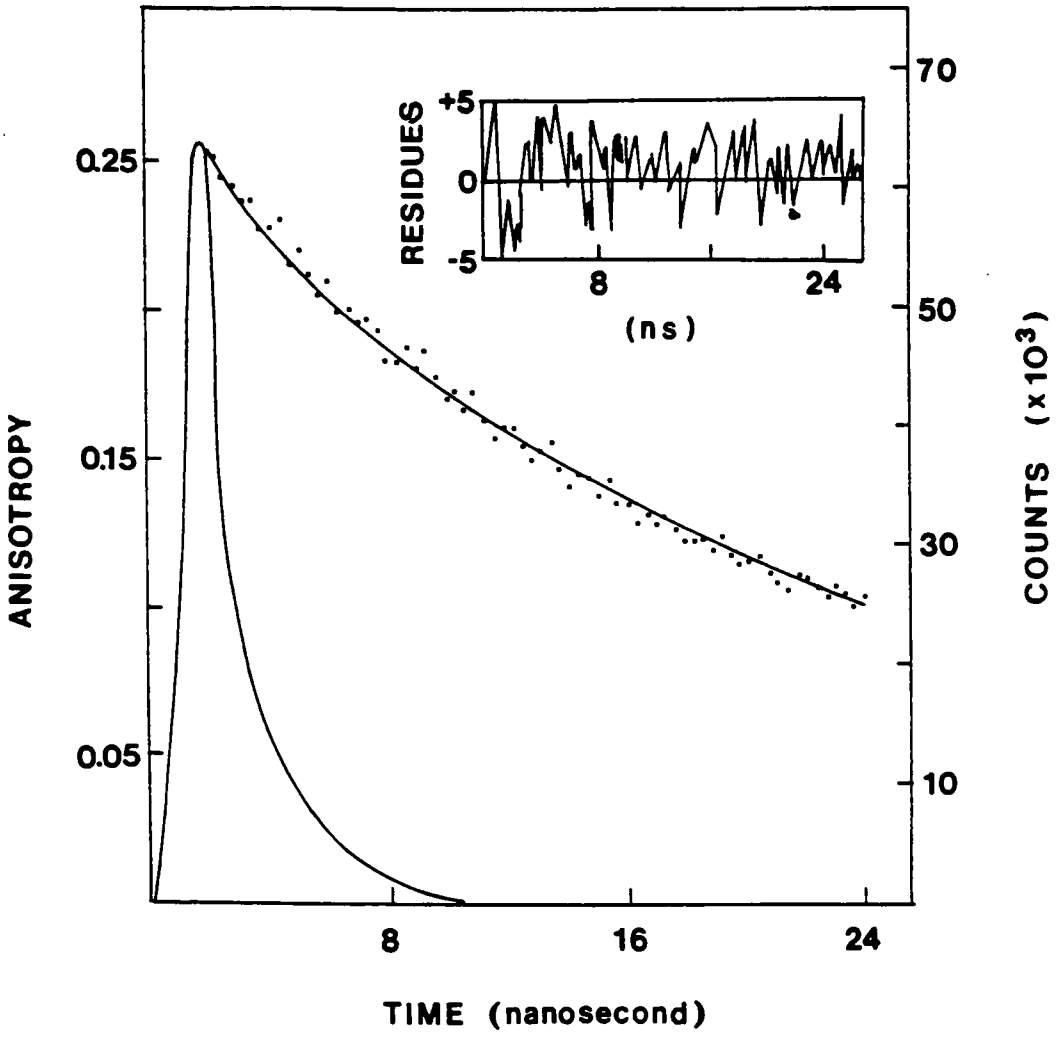



**Fig. 5-1.** *Anisotropy Decay Function of AEDANS-myo-Inositol Monophosphatase at a concentration of 6uM in 50 mM Tris·HCl buffer (pH 7.5) .*

The best fit parameters are $A_o$ = 0.25 (limiting time anisotropy) and $\phi$ = 30 ns (■). The extrapolated value of the anisotropy function $A(t)$, $A_D$ = 0.26 is smaller than the theoretical value $A_D$ = 0.30 expected for a chromophore rigidly bound to the protein. Including is the plot of the residuals corresponding to the deconvoluted data fitted to a single exponential function. Excitation wavelength 340 nm, emission was selected using glass filter (C.S.-3-72, Corning). The lamp profile (curve) is included.

the dimeric structure (O'Connor & Phillips, 1984).

Gel Filtration Chromatography

Further evidence for the presence of stable dimeric structures at protein concentrations lower than 1 *u*M was derived from gel filtration chromatography studies.

As shown in Fig. 5-2, the elution profile of inositol monophosphatase exhibits a symmetrical peak characterized by molecular mass of 58 kDa. Moreover, the elution profile of the enzyme is not influenced by concentrations of Gu·HCl ranging 1 to 2 M (Fig. 5-2). A significant change in the elution profile was detected at Gu·HCl concentrations approaching 4 M, but the absence of a second peak or shoulder in the elution diagram makes it difficult the assignment of retention times corresponding to unfolded monomer and folded monomer. At Gu·HCl concentrations above 4 M, the protein lost most of its secondary structure, and probably exists as an unfolded monomer. In this connection, it is interesting to note that it has been reported that bovine brain inositol monophosphatase is completely dissociated in 8 M urea (Gee et al., 1988).

Reversible Denaturation of Inositol Monophosphatase

Samples of inositol monophosphatase (17 *u*M), incubated with increasing concentrations of Gu·HCl in 10 mM Tris·HCl buffer (pH 7.5) for 1 hour at 25 °C, were diluted 100 fold into the assay mixture containing $\beta$-glycerophosphate (2 mM) and $MgCl_2$ (20 mM) in 10 mM Tris·HCl buffer (pH 7.5) and the amount of *Pi* released determined colorimetrically as outlined in "Experimental Procedures". After the dilution of the Gu·HCl treated samples, the catalytic activity of the monophosphatase was found to be completely restored as indicated by the results of Fig 5-3. When the enzyme was assayed in the presence of increasing concentration of Gu·HCl, it was found that concentrations of the denaturant of approximately 1 M abolished 85 % of the original catalytic activity.

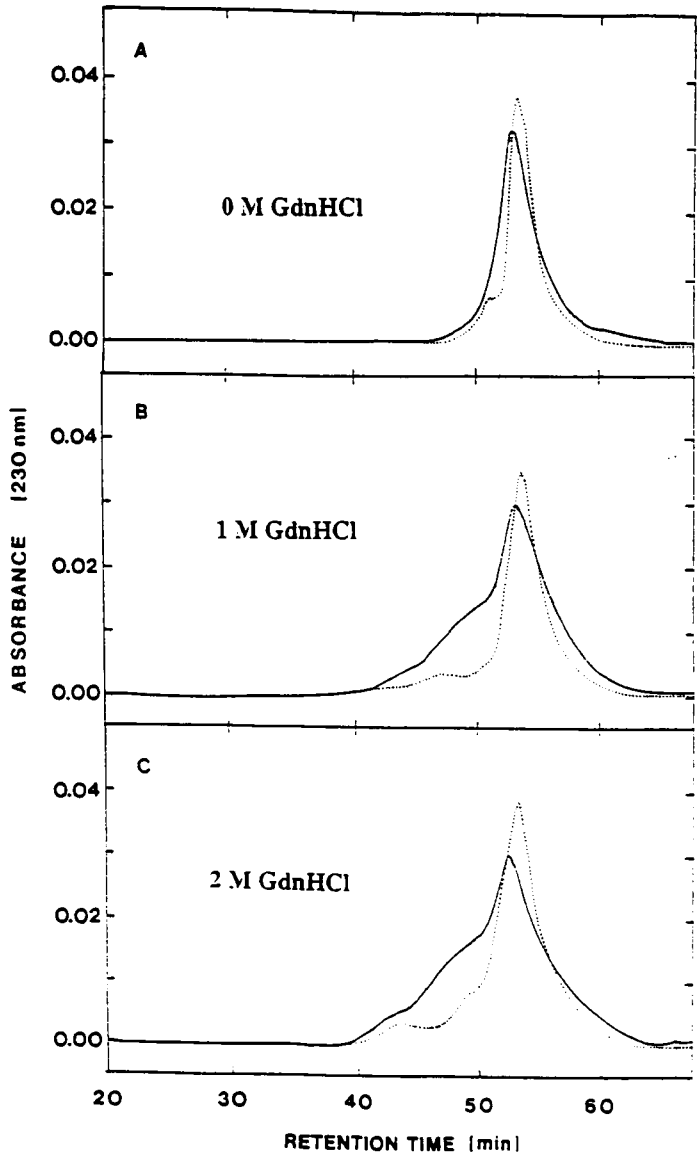



**Fig. 5-2.** *Elution Profile of myo-Inositol Monophosphatase by HPLC Analysis.* The enzyme was chromatographed on a TSK 3000 SW column (600 x 7.5 mm) at a flow rate of 0.2 ml/min and a pressure of 1.1 MPa; the mobile phase was 10 mM Tris·HCl (pH 7.5) containing various concentrations of Gu·HCl. Standards of known molecular weight were chromatographed under similar conditions. The elution profile of the enzyme (–) in the presence of 0, 1 and 2 M Gu·HCl are given in panels A, B and C, respectively, together with the elution profile of ovalbumin (45kDa) (···) in the presence of increasing concentrations of Gu·HCl.

Reversible Unfolding

The effect of Gu·HCl on the structural changes of inositol monophosphatase was investigated by means of circular dichroism, emission anisotropy and fluorescence spectroscopy.

The unfolding of the enzyme was studied by CD spectroscopy at 220 nm, a measure of the α-helix content of proteins (Jaenicke, 1984). All measurements were performed after incubation of the protein solution for 1 hour at 25°C in the presence of increasing concentrations of Gu·HCl. Control measurements showed no change of the signal after 1 hour incubation at 25°C.

The recorded spectra included in Fig. 5-4A indicate that the protein in 1 M Gu·HCl exhibited a good deal of secondary structure and that some residual dichroism was still observed at 4M Gu·HCl. Fig. 5-4B shows how the mean residue ellipticity, $[\theta]$, decreased reversibly as a function of the Gu·HCl concentration.

For the estimation of the change in free energy ($\Delta G$) associated with unfolding, a two state model was assumed in the analysis of reversible denaturation ;

$$F \rightleftharpoons 2U$$

it is assumed that inositol monophosphatase (F) exists as a folded dimer in equilibrium with an unfolded monomer (U). The equilibrium constant, $K_{eq}$, is related to the protein concentration in monomers ($P_o$) and to the fraction ($f$) of inositol monophosphatase in the folded state by the equation ;

$$K_{eq} = 2 \cdot P_o \cdot (1-f)^2 / f \qquad (6)$$

$f$, the fraction of folded enzyme is determined from the mean residual ellipticity measured at 220 nm.

$$f = ( \theta - \theta_{unf}) / ( \theta_{fol} - \theta_{unf}) \qquad (7)$$

The free energy of unfolding $\Delta G = - RT \ln K_{eq}$ was found to vary linearly with Gu·HCl concentration; therefore $\Delta G$ ($H_20$) = 11.5 kcal/mol was obtained by extrapolating to zero Gu·HCl concentration.

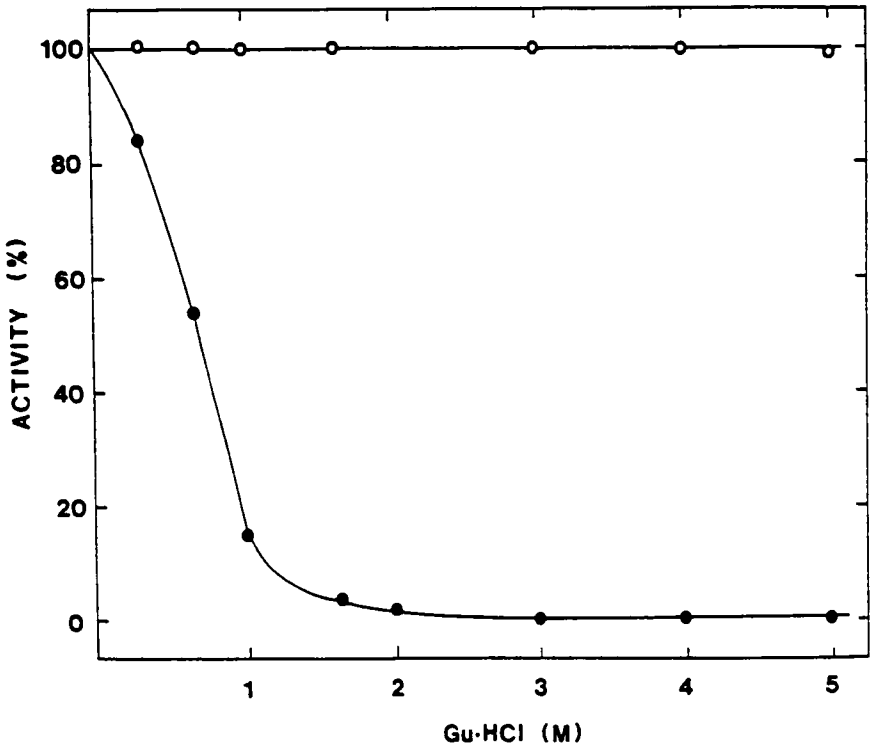
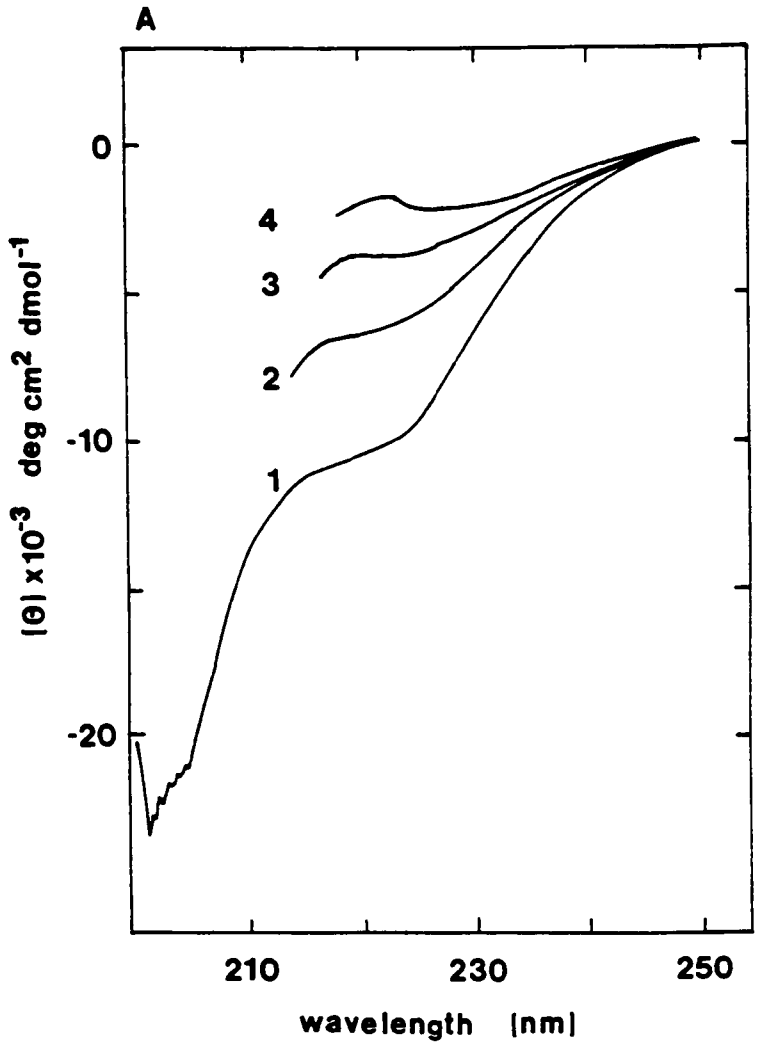



**Fig. 5-3.** *Reversibility of the Denaturation of myo-Inositol Monophosphatase by Gu·HCl.*

Results obtained with samples of the enzyme (17 $u$M) incubated with increasing concentrations of Gu·HCl in 10 mM Tris·HCl buffer (pH 7.5) for 1 hour at 25°C and diluted 100 fold into the assay mixture (o). Samples of enzyme assayed in the presence of increasing concentrations of Gu·HCl (•). The assay mixture contains $\beta$-glycerol phosphate (2 mM), $MgCl_2$ (20 mM) in 10 mM Tris·HCl buffer (pH 7.5).


A
0
-10
-20
4
3
2
1
210
230
250
wavelength [nm]
[θ] x10^-3 deg cm^2 dmol^-1

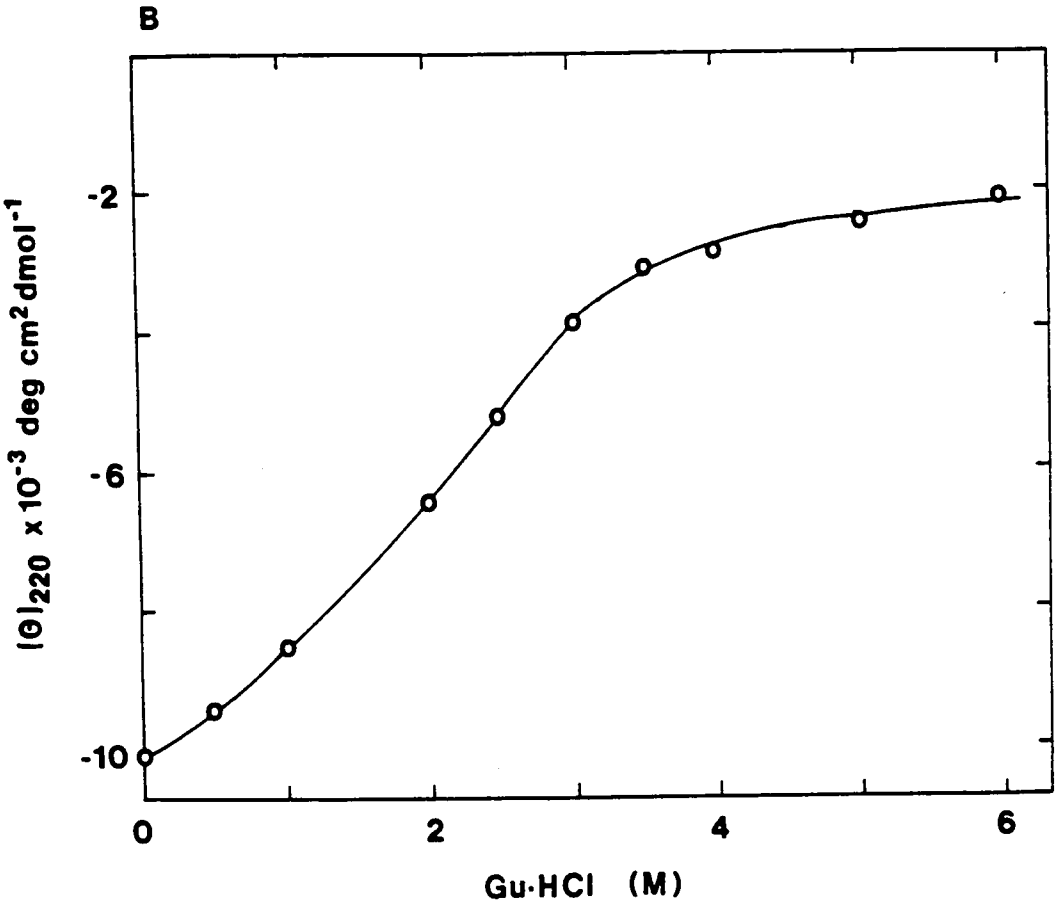



**Fig. 5-4.** *Changes in Circular Dichroism of myo-Inositol Monophosphatase in the Presence of Increasing Concentrations of Gu·HCl.*

(A) Circular dichroism spectra of the enzyme (13.5*u*g/ml) recorded in 10 mM Tris·HCl (pH 7.5) containing various concentration of Gu·HCl. Curve 1 represents the CD spectrum of the native enzyme; curve 2, in the presence of 2M Gu·HCl; curve 3, 3 M Gu·HCl; curve 4, 6M Gu·HCl. All measurements were performed after incubation of the protein solution for 1 hour at 25 °C.

(B) Gu·HCl denaturation curve for *myo*-inositol monophosphatase in 10 mM Tris·HCl (pH 7.5) at 25°C. The mean residue ellipticity was recorded at 220 nm as a function of Gu·HCl concentration.

*myo*-Inositol monophosphatase contains 3 tryptophanyl residues/monomer (Diehl, 1990) and upon excitation at 295 nm shows a structureless emission band centered at 338 nm (Fig. 5-5). Addition of increasing concentrations of Gu·HCl brings about a red shift in the band position of the emission spectrum together with a decrease in the fluorescence intensity emitted at 340 nm. Perturbation of the maximum emission occurs at Gu·HCl concentrations above 2 M as shown by the spectra recorded in Fig. 5-5. The exposure of tryptophanyl residues to solvents on unfolding is the major factor that contributes to the observed red shift in the protein emission.

The results of steady state emission anisotropy measurements of samples of *myo*-inositol monophosphatase treated with Gu·HCl under experimental conditions identical to those used in the circular dichroism studies are shown in Fig. 5-6. As the tryptophanyl residues of the enzyme become exposed to the solvent, their rotational mobility increases and the emission anisotropy decreases in the manner depicted in Fig. 5-6. Like in the circular dichroism measurements, a small change in the emission anisotropy is detected at Gu·HCl concentration of 1 M, suggesting that the dimeric structure is compact and retains a large portion of the secondary structure. The emission anisotropy reaches a minimum value at concentrations above 4 M Gu·HCl when the unfolded monomer is the predominant species in solution. The process of inositol monophosphatase unfolding as monitored by emission anisotropy measurements occurs as a single cooperative transition, indicating an equilibrium between folded dimer and unfolded monomer. The parameters calcutated from different methods for GdnHCl induced unfolding of monophosphatase is shown in Table 5-2.

**TABLE 5-2**

*Parameters for GdnHCl induced unfolding of IMPase*

*as determined by different methods*

| | Cm | $\Delta G(H_2O)$ (kcal/mol) |
|---|---|---|
| CD | 2.1 | 11.5 |
| Fluorescence | 2.1 | 11.0 |
| Anisotropy (4 *u*M) | 2.5 | 11.7 |
| (1 *u*M) | 2.5 | 12.3 |

Cm is GdnHCl concentration of denaturation midpoint

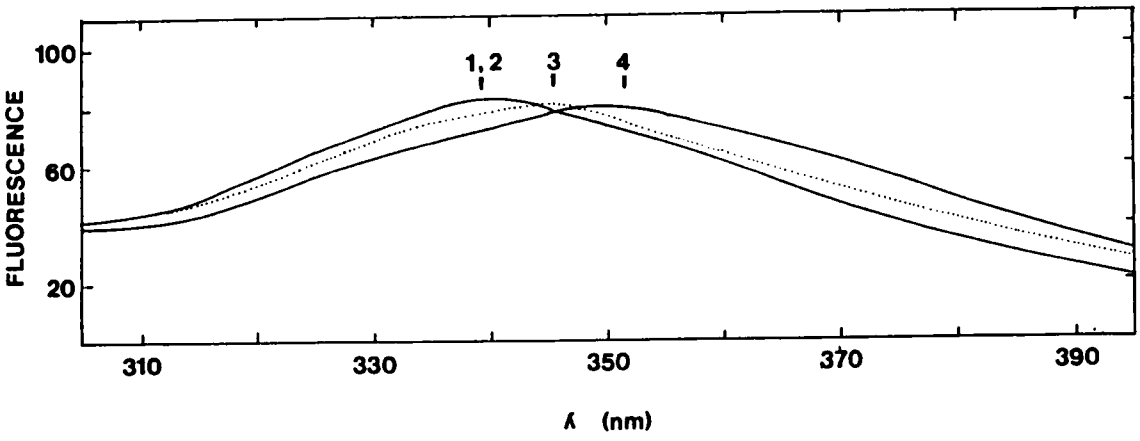



**Fig. 5-5.** *Emission Spectra of myo-Inositol Monophosphatase in Various Concentration of Gu·HCl.*

The spectra recorded in the presence of 0 (curve 1), 1M (curve 2), 3M (curve 3) and 4M Gu·HCl (curve 4). The absorbance of the samples at the maximum of excitation 295 nm were 0.1. The samples were treated with Gu·HCl for 1 hour at 25°C prior to recording the emission spectra.

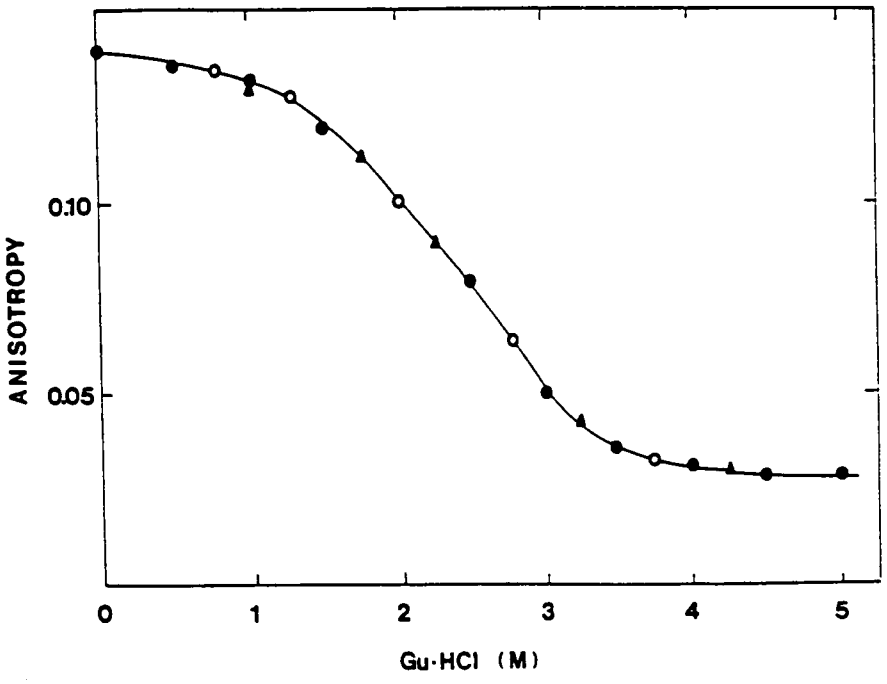



**Fig. 5-6.** *Dependence of Emission Anisotropy of myo-Inositol Monophosphatase on Gu·HCl Concentration at 25°C.*

Concentrations of the enzyme were 4 *u*M (o) and 1 *u*M (▲) in 10 mM Tris·HCl (pH 7.5). Filled circles (•) represent results obtained when samples kept in Gu·HCl (5M) were diluted with Tris·HCl buffer and the anisotropy determined after 15 min incubation. Final concentration of the protein was 4 *u*M. Excitation set at 295 nm.

# DISCUSSION

*myo*-Inositol monophosphatase is a key enzyme of the inositol phosphate second messenger pathway. It provides inositol which is required for synthesis of phosphatidyl inositol. Like many other proteins (Jaenicke, 1984; Pace *et al.*, 1991), *myo*-inositol monophosphatase undergoes a cooperative structural transition in solution containing Gu·HCl over the concentration range 1-6 M. This transition alters the secondary structure of the protein as indicated by changes in the peptide circular dichroism at 220 nm, and the tertiary structure as indicated by changes in the degree of exposure of tryptophanyl residues and by changes in the emission anisotropy.

The circular dichroism and emission anisotropy measurements yield similar estimates of the dependence of the equilibrium on Gu·HCl concentrations. It is generally accepted that the kinetic attainment of the folded native state does not depend on a random search, and a limited number of pathways exist involving kinetic intermediates between the fully unfolded and native states (Kim & Baldwin, 1982; Christensen & Pain, 1991; Ptitsyn, 1991).

The major question posed by our studies concerns the nature of the intermediate states in the reversible denaturation of *myo*-inositol monophosphatase. The enzyme is a dimer of 58 kDa characterized by a rotational correlation time of 30 ns corresponding to compact dimeric structure. The presence of a compact folded monomer intermediate could not be detected by time emission anisotropy measurements of a sample exposed to 1 M Gu·HCl and dialyzed against buffer. Moreover, the dimeric structure is stable at concentrations below 1mM when exposed to 1 and 2 M Gu·HCl. Indeed, the elution patterns obtained after gel filtration chromatography indicate the existence of dimeric species. A broad peak was detected when the gel filtration studies were conducted in the presence of 4 M Gu·HCl (results are not shown), but it was virtually impossible to

identify compact monomers in equilibrium with unfolded monomers. It is worthy of note that under this set of experimental conditions, the protein has lost most of the secondary structure and the tryptophanyl residues are completely exposed to the solvent.

The possibility exists that a small population of the protein consists of folded monomers, but our experimental studies failed to detect those molecular species along the unfolding pathway. For the above mentioned reasons, we have described the denaturation mechanism of *myo*-inositol monophosphatase as a two state process, *i.e.*, equilibrium between folded dimer and unfolded monomer. This model has been extensively developed in the case of small monomeric proteins (Privalov *et al.*, 1986) as well as small dimeric proteins (Pakula & Sauer, 1989; Liang & Terwilliger, 1991) for which a simple sigmoidal transition curve should be expected. One of the most interesting features of the present studies is the finding that the denaturation of *myo*-inositol monophosphatase in the presence of Gu·HCl is a reversible process. The catalytic activity of the enzyme is restored after diluting the unfolded form of the protein with the substrate.

Judging from the results of the enzymatic assays, it appears that there is no significant delay in the reactivating process. Therefore, it is pertinent to ask the following question: what are the rate limiting steps in the folding process leading to the formation of stable dimeric structures ?. One possibility is that dimerization and the formation of tertiary structure are dependent upon each other and occur simultaneously. Alternatively, the slower dimerization may occur first, followed by a faster rearrangement of the tertiary structure. Studies on the kinetics of folding, monitored by fluorescence and emission anisotropy measurements, can help to distinguish between the alternative models.

# CHAPTER 6

# INTERACTION OF 70 kDa HEAT SHOCK COGNATE PROTEIN WITH PEPTIDES AND *myo*-INOSITOL MONOPHOSPHATASE

## INTRODUCTION

Members of the stress-70 family have been implicated in the stabilization of unfolded proteins before assembly in the cytosol (Beckman, 1990), in rearrangement of protein oligomers (Rothman & Schmid, 1986) and in the dissolution of high molecular weight aggregates (Pelhman, 1986). The interaction of 70 kDa heat shock cognate protein (Hsc 70) with unfolded proteins, *i.e.*, reduced carboxymethylated $\alpha$-lactalbumin, reduced carboxymethylated RNase and apocytochrome C, has been investigated by HPLC chromatography (Palleros *et al.*, 1991) and by measuring the enhanced weak ATPase activity of the chaperon protein (Sadis & Hightower, 1992).

Hsc 70 also binds small molecular weight peptides with a concomitant enhancement of its ATPase activity (Flynn *et al.*, 1991). Despite those interesting studies, there is little information available on the actual values of the dissociation constants characterizing the protein-Hsc 70 complexes. Consequently the magnitude of the free energy associated with the binding process is unknown.

The aim of the present work is two-fold ; first, to study the interaction of small molecular weight peptides with bovine Hsc 70 by protein fluorescence measurements, and, second, to investigate the interaction of bovine Hsc 70 with folded and unfolded conformations of the enzyme inositol monophosphatase by means of steady-state emission anisotropy. The cytosolic enzyme inositol monophosphatase is suitable for these

studies because the protein undergoes a reversible process of dissociation and unfolding in the presence of guanidine HCl (Kwon et al., 1993).

## EXPERIMENTAL PROCEDURES

### Materials

RNase-S-peptide, octapeptide of Sickle cell and ATP-agarose were obtained from Sigma. [$\gamma$-$^{32}$P]ATP (>3000 Ci/mmol) from ICN Radiochemicals. Charcoal was obtained from Aldrich. The standard buffer for all the experiments presented in this paper, except where stated, is 20 mM Hepes NaOH (pH 7.0), 2 mM Mg Acetate, 25 mM KCl, 10 mM $(NH_4)_2$ $SO_4$ and 0.8 mM DTT. and is hereafter referred to as the standard buffer.

### Enzyme Purification

Hsc 70 protein from bovine brain cytosol was purified as described by Schlossman *et. al.* (1984) and Sadis *et. al.* (1990). Dialyzed crude cytosol was prepared as described (Schmid & Rothman, 1985) and ATPase activity was measured for each purification step by the continuous spectroscopic assay described in "enzymatic assays". The key step in the purification of the protein relied on the ability to bind to ATP-agarose under high salt concentrations and its subsequent elution with millimolar concentration of ATP. Using a combination of DEAE-cellulose chromatography and ATP-agarose affinity chromatography, Hsc 70 preparations were greater than 95% pure as judged by SDS-PAGE stained with Coomassie blue R-250. From 500g of brain tissue, approximately 15 mg of Hsc 70 was obtained. One-dimensional SDS-PAGE was performed as described by Laemmli (1970).

The procedure developed by Meek *et. al.* (1988) for the purification of bovine brain *myo*-inositol monophosphatase was used in the isolation of the enzyme from pig

brain. The major purification steps are (a) removal of interfering proteins by heat denaturation at 75 °C for 1 hr, (b) separation by DEAE-Sephacel at pH 6, (c) adsorption of remaining protein on a procion Red-Sepharose column and (d) chromatography on mono Q anion exchange column. The specific activity of the purified enzyme was 3 *u*mol / min. mg. with D,L,$\beta$-glycerophosphate at pH 7.5, 25 °C. Protein concentration was determined by the methods of Bradford (1976). The specific ATPase activity of purified Hsc 70 is 1.2 nmol/min.mg.

Enzymatic Assays

ATPase assays for Hsc 70 were performed exactly as described by Bias (1975) and Jackson *et. al.* (1993). The protein was mixed with 0.5 mM ATP containing 1 *u*Ci of [$\gamma$-$^{32}$P]ATP in the standard buffer. Aliquots were withdrawn at given time intervals and mixed with an acidified suspension of activated charcoal, which served to adsorb unhydrolyzed nucleotide and protein material. The suspension was then centrifuged and the amount of [$^{32}$P]*Pi* hydrolyzed in the supernatant was determined by liquid scintillation counting. The cpm data were converted to picomoles after correction for background activity derived from spontaneously hydrolyzed phosphate.

Inorganic phosphate, released by *myo*-inositol monophosphatase, was assayed with malachite green as described by Lanzetta *et al.* (1979) and by a continuous spectrophotometric assay in which free *Pi* reacts with inosine to give hypoxanthine and ribose-1-phosphate (catalyzed by nucleoside phosphorylase) as described by Gee at. al. (1988). Xanthine oxidase catalyzes the reduction of a dye; *i.e.*, 2-P-iodophenyl-3-P-nitrophenyl-5-phenyltetrazolium, to give a formazan detected spectrophotometrically at 546 nm ($\varepsilon = 1.84 \times 10^4$ $M^{-1}cm^{-1}$). Each assay contained 50 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 1 mM inosine, 0.5 mM tetrazolium dye, 1 mM D,L-$\beta$-glycerophosphate, 0.11 unit of nucleoside phosphorylase, 0.04 unit of xanthine oxidase in a final volume of 1 ml. The auxiliary enzymes, xanthine oxidase from cow milk and calf spleen nucleoside

phosphorylase, were purchased from Boehringer and dialyzed against several change of 50 mM Tris-HCl (pH 7.5) to remove contaminating *Pi*. Aliquots of both enzymes were stored at -20 °C.

The other enzymatic assays were performed with reaction in 50 mM Tris-HCl buffer (pH 7.5) containing $\beta$-glycerophosphate (1 mM), $MgCl_2$ (1 mM) and NaCl (0.1 M). At given time incubation, 50 *u*l of sample aliquots were withdrawn and mixed with 1ml of reaction mixture containing malachite green/ammonium molybdate. After incubation for 30 min at 25 °C, the color developed was read at 660 nm in spectrophotometer.

Labeling of Inositol *myo*-Inositol Monophosphatase

The labeling of inositol monophosphatase was conducted with EDANS as described by Kwon *et al.* (1993). The enzyme at a concentration of 5 mg/ml was allowed to react with 1,5-I-EDANS in 0.1 M Hepes buffer (pH 7) at 4 °C. The reaction was allowed to proceed for 5 hrs. Excess free reagent was removed by dialysis, followed by gel filtration through a Sephadex G-25 column equilibrated with same buffer. The degree of labeling of the enzyme was determined spectrophotometrically using an extinction coefficient of $6.2 \times 10^3\ cm^{-1}M^{-1}$ at 340 nm. The incorporation of 1.2 mol dye/dimer does not affect the catalytic function of the enzyme.

Spectroscopy

Absorption spectra were recorded with a Shimadzu UV-160 spectrophotometer. Fluorescence emission spectra were obtained with the use of a Perkin Elmer LS-50B spectrofluorometer. The excitation and emission slit width were set at 2.5 nm.

Steady state emission anisotropy measurements were performed in a SLM double beam polarization apparatus equipped with a Racal-Danan digital multimeter. Illumination provided by a Xenon lamp (200watts) was passed through a grating

monochromator (Bausch & Lomb Inc.). Fluorescence emission light was filtered through Corning glass filters (C-S-3-72).

## RESULTS

### Emission Spectra of Hsc 70

Steady-state fluorescence spectroscopy has been used to obtain information on dynamic properties of biological macromolecules, the parameters of fluorescence emission, *i.e.*, position, shape and intensity, are dependent on the electronic and dynamic properties of the chromophore environment. Dynamic properties of proteins, which can also be deduced from steady-state measurements, are associated with the phenomenon called red-excitation effect (Galley & Purkey, 1970; Demchenko, 1986).

Site selective excitation at the red-edge of the absorption band photoselects chromophores with minimal electronic transition frequencies. The corresponding instantaneous emission spectrum will be shifted to long wavelengths relative to the spectrum observed at main band excitation.

According to accepted theories (Mazurenko, 1986; Demchenko, 1982), there is a relationship between the mean-wavenumber of the emission spectrum ($\bar{\nu}$), the wavenumber of the emission spectrum excited at the red-edge of the absorption band ($\bar{\nu}^{edge}$), the fluorescence lifetime ($\tau_f$), and the dipolar relaxation time ($\tau_r$).

$$\frac{\bar{\nu} - \bar{\nu}^{edge}}{\bar{\nu}_o - \bar{\nu}_o^{edge}} = \frac{\tau_r}{\tau_r + \tau_f} \qquad (1)$$

where $\bar{\nu}_0$ is the mean frequency of the unrelaxed spectrum; typically observed at low temperature when the motion of the dipoles of the chromophore environment is restricted; *i.e.*, $\tau_r > \tau_f$.

Since the red-edge effect has been detected in proteins exhibiting maximum of emission within the range 325-340 nm (Wasylenski et al., 1992), we have decided to examine the emission properties of Hsc 70 as a function of the excitation wavelength. Hsc 70 is a protein composed of a single polypeptide chain folded into two major domains (Milarski & Morimoto, 1989). One tryptophan residue is located in a highly conserved domain, whereas the second tryptophan residue is positioned in a less conserved C-terminal domain.

The dependence of the position of the spectral emission maximum as a function of excitation wavelength is shown in Fig. 6-1. Clearly, the maximum of emission of the tryptophan residues remains invariant upon variation of the excitation wavelength. The absence of any spectral shift upon red-edge excitation is interpreted to mean that the tryptophan residues of Hsc 70 are exposed to the rapidly relaxing aqueous solvent. It should be noted that addition of ATP, a substrate of Hsc 70, has no effect on the band position of the protein fluorescence.

Further information on the degree of exposure of the tryptophan residues to collisional encounters with small molecules was derived from fluorescence measurements under conditions of acrylamide quenching. Fig. 6-2 shows a typical Stern-Volmer plot for Hsc 70 at 20 °C obtained with excitation at 295 nm. The plot is a straight line; it does not show any downward curvature characteristic of proteins possessing tryptophan residues differing in their accessibility to collisional quenchers (Wasylenski, 1992). Hence, two different experimental approaches indicate that the tryptophan residues of Hsc 70 are exposed to the rapidly relaxing aqueous solvent.

Binding of Peptides to Hsc 70

The interaction of two peptides with Hsc 70 was monitored by measuring the fluorescence emitted by the protein. The peptides tested in our studies were an octapeptide, Val-His-Leu-Thr-Pro-Val-Glu-Lys, corresponding to the amino terminal

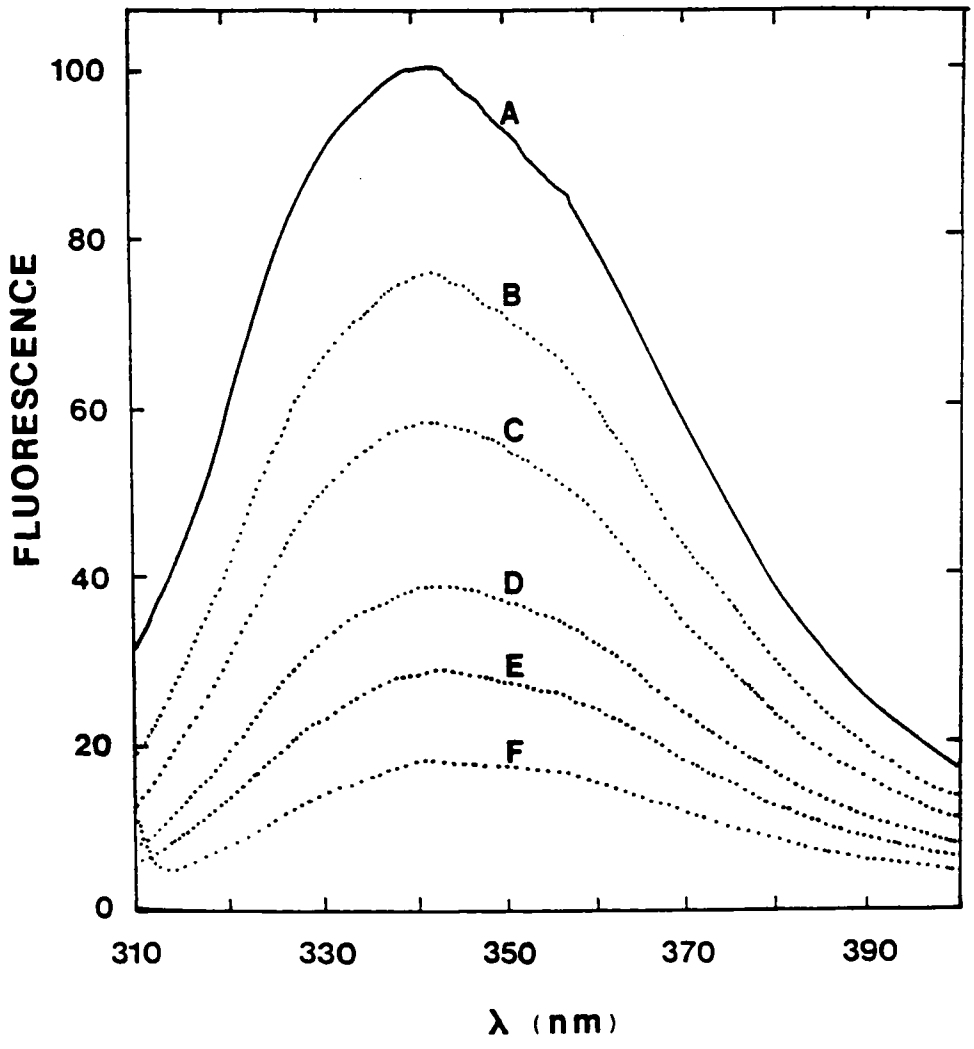



**Fig. 6-1.** *Influence of the excitation wavelengths on fluorescence emission spectra of Hsc 70 in standard buffer at 20 °C.*

The data for excitation wavelengths at 290 (curve A), 293 (B), 295 (C), 298 (D), 300 (E) and 303 nm (F) reveal no differences of the positions of the fluorescence maxima.

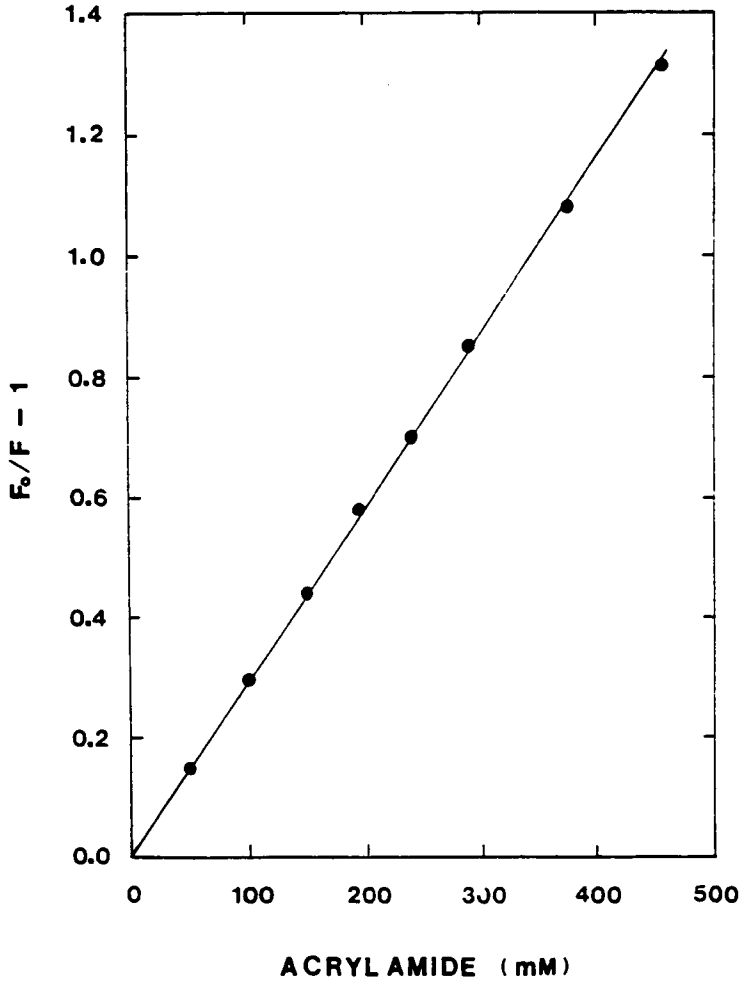



**Fig. 6-2**. *Stern-Volmer plot for acrylamide quenching of Hsc 70.*

Experiments performed at 20 °C with excitation at 295 nm and emission at 340 nm. The solid line represents the least-square fit with the parameter $K_{sv} = 3.0\ M^{-1}$.

region of the $\beta$-chain of sickle cell hemoglobin, and a twenty amino acids peptide (RNase S-peptide) derived from limited proteolysis of ribonuclease (Richaros & Vithayathil, 1959).

The emission band of Hsc 70, excited at 295 nm, is quenched by the addition of either RNase S-peptide or the octapeptide in the manner depicted in Fig. 6-3. The fraction of binding sites (a) occupied by the peptide was determined with the aid of equation (2).

$$\alpha = \frac{F_o - F}{F_o - F_m} = \frac{[E - Peptide]}{E_t} \qquad (2)$$

where F is the observed fluorescence when both free and bound peptide are in equilibrium; $F_0$ is the fluorescence intensity of the protein and $F_m$ is the fluorescence observed at peptide concentrations required to saturate the protein. For one binding site per protein molecule, the fraction of occupied binding sites ($\alpha$) is related to the dissociation constant $K_d$ by equation (3).

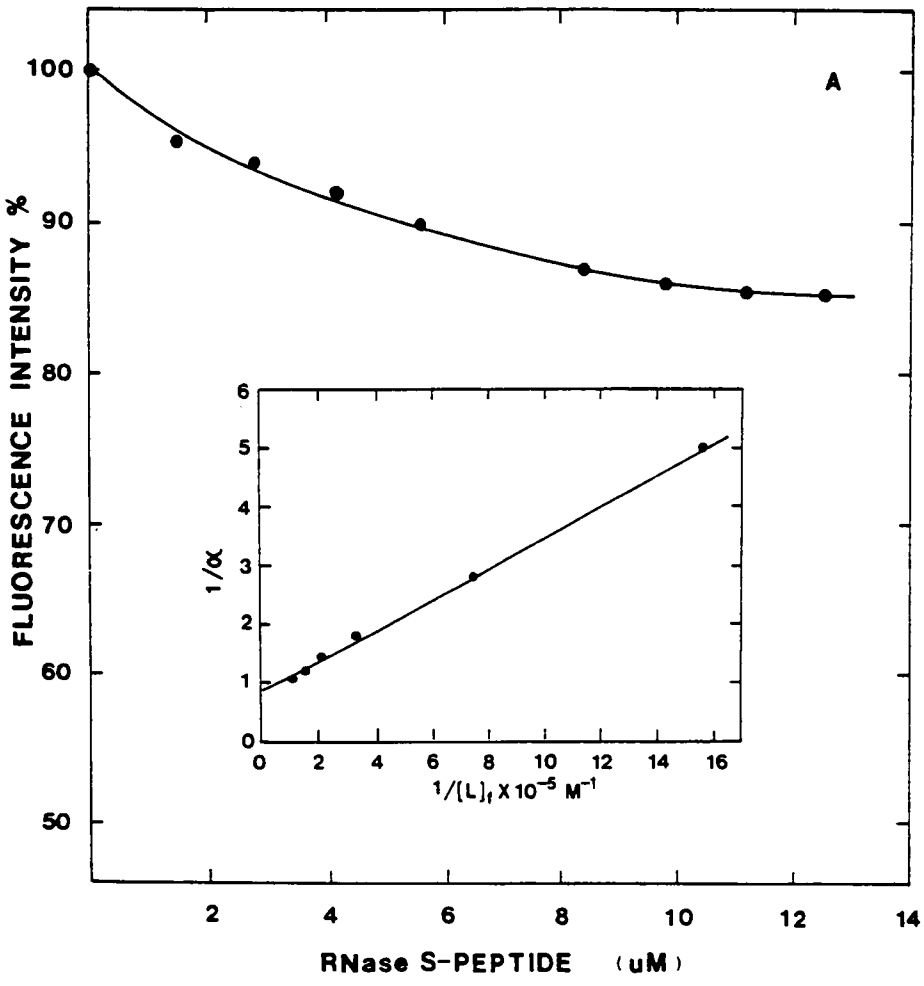
$$\frac{\alpha}{1-\alpha} = \frac{[Peptide]_{free}}{K_d} \qquad (3)$$

From the results of Fig. 6-3, dissociation constants of 2.7 $u$M and 9.3 $u$M were obtained for Hsc70 - (RNase S-peptide) and Hsc70-octapeptide, respectively (Table 6-1).

ATPase Activity of Hsc 70

Purified Hsc 70 is endowed with low ATPase activity (1.2 nmol/min mg). To test whether the RNase S-peptide was capable of stimulating ATPase activity, the hydrolysis of ATP catalyzed by Hsc 70 was measured in the presence of the peptide.

The results of typical experiments that measures the release of [$^{32}$P] *Pi* from the substrate [$\gamma$-$^{32}$P] ATP at 37 $^{o}$C are given in Fig 6-4. The addition of RNase S-peptide to Hsc 70 increases the specific activity of the enzyme 1.4 fold. In marked contrast to the peptide, ribonuclease does not enhance the catalytic activity of Hsc 70.


A
FLUORESCENCE INTENSITY %
100
90
80
70
60
50
2
4
6
8
10
12
14
RNase S-PEPTIDE (uM)
1/α
0
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2
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16
1/[L]f X 10⁻⁵ M⁻¹

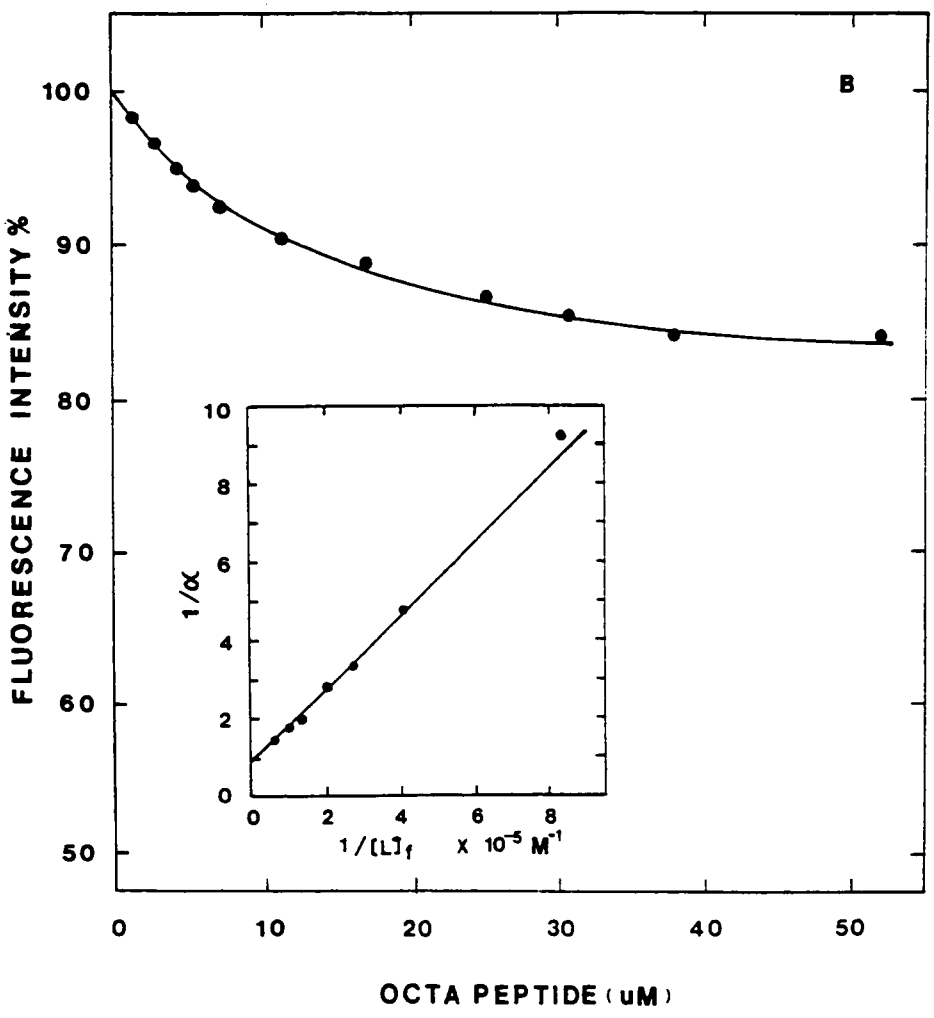



**Fig. 6-3.** *Changes in the fluorescence intensity of Hsc 70 (1.8 uM) on titration with increasing concentration of RNase S-Peptide (A) and octapeptide (B).*

Apparent dissociation constant values were obtained by plotting $1/\alpha$ against $1/[\text{peptide}]_f$ (*inset figures*) in the analysis of the data. It is assumed that the stoichiometry of the complex is 1:1. The dissociation constants ($K_d$) of 2.7 and 9.3 $u$M were obtained for RNase S-peptide and the octapeptide complexed to Hsc 70, respectively. Experiments conducted in the standard buffer at 20 °C with excitation wavelength at 295 nm.

**Table 6-1**

Apparent Dissociation Constant Values ($K_d$) of Hsc 70 bound to Peptides

| Sample | $K_d$ (uM) | $\Delta$G (kcal/mole) |
|---|---|---|
| RNase S-peptide | 2.7 | - 7.45 |
| Octapeptide * | 9.3 | - 6.67 |
| EDANS-IMPase | 1.2 | - 7.92 |

$\Delta$G is the apparent standard free energy of association.

* The amino terminal region of the $\beta$-chain of sickle cell hemoglobin.

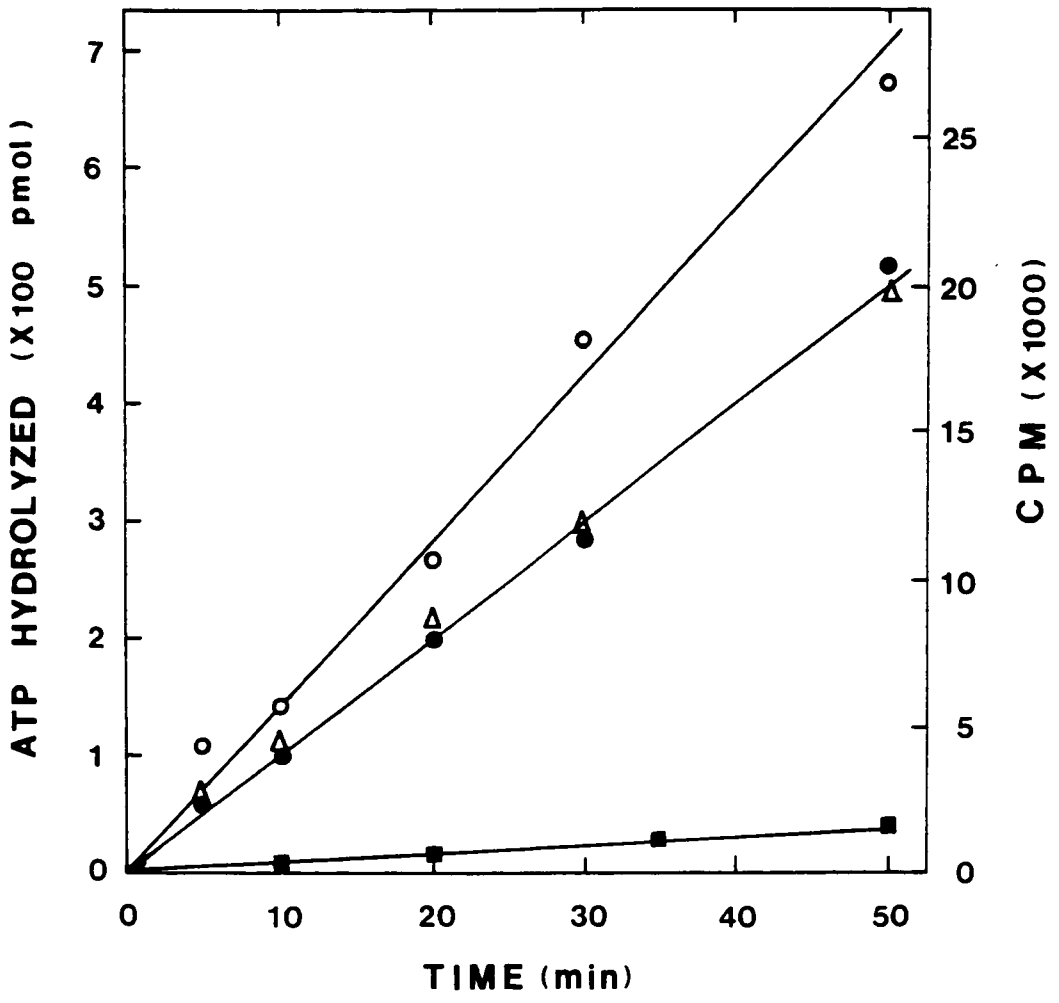



**Fig. 6-4.** *ATP hydrolysis and the effect of RNase and S-peptide.*

ATPase activity was measured in reactions containing 0.5 mM ATP, and 2 *u*Ci of [$\gamma$-$^{32}$P] ATP in the standard buffer at 37 °C. RNase (Δ) or S-peptide (o) were added to a final concentration of 100 *u*M and 25 *u*M, respectively. Aliquots were withdrawn and the amount of ATP hydrolyzed was measured as described in "experimental procedure". Control experiments were also performed to measure the spontaneous ATP hydrolysis in the absence of Hsc 70 (■) and the intrinsic rate of ATPase activity by Hsc 70 alone (100 *u*g, •).

Binding of a Cytosolic Protein to Hsc 70

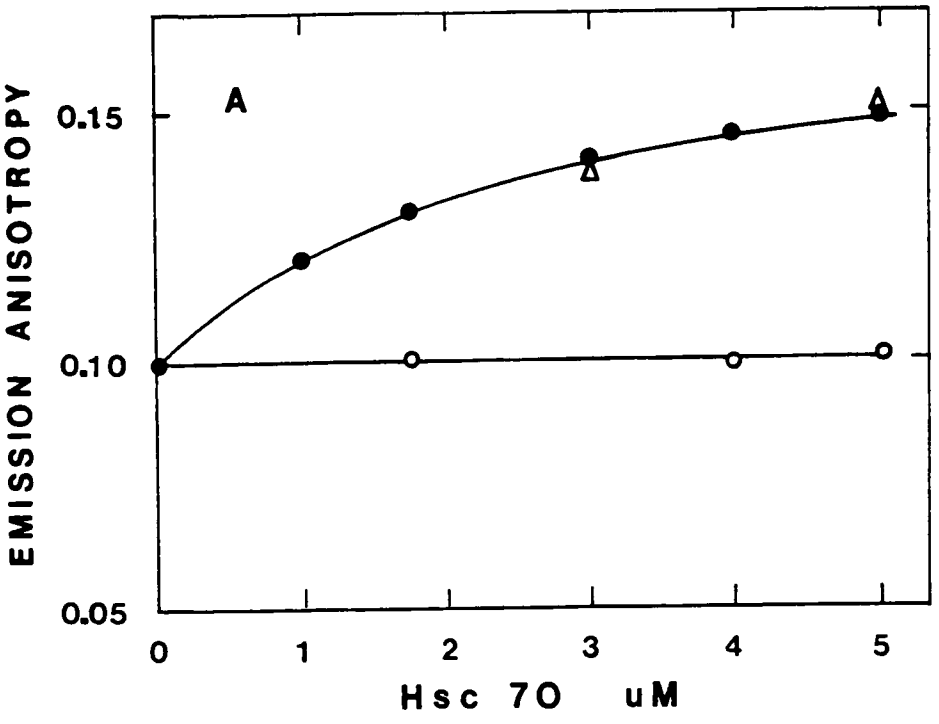
The binding of small molecular weight peptides to Hsc 70 could be assessed by direct measurements of protein fluorescence because the peptides tested did not absorb in the spectral region coinciding with the absorption of tyrosine and tryptophan residues of the protein. In order to study the binding of proteins to Hsc 70, it is necessary to label the proteins with extrinsic probes characterized by spectroscopic properties distinct from those of natural chromophores, *i.e.*, tyrosine and tryptophan.

Inositol monophosphatase tagged with the fluorescent probe, EDANS, retains its catalytic activity. Moreover, the protein undergoes a reversible process of dissociation and unfolding upon treatment with 4 M guanidine HCl (Kwon et al., 1993), which permits the investigation of the binding of unfolded conformations to Hsc 70.

The spectroscopic properties of EDANS-monophosphatase, *i.e.*, fluorescence emission and emission anisotropy, remain constant after addition of increasing concentrations of Hsc 70 to a fixed concentration of tagged enzyme (1 *u*M) shown in Fig. 6-5. Under this set of experimental conditions, the formation of a stable complex between folded inositol monophosphatase and Hsc 70 was not detected.

In order to measure the affinity of the unfolded conformation of inositol monophosphatase for Hsc 70, samples of EDANS-monophosphatase (20 *u*M) were allowed to unfold in the presence of 4 M guanidine HCl at 20 °C for 30 minutes, diluted twenty fold with solution of increasing concentrations of Hsc 70 in 50 mM Hepes buffer (pH 7), and their emission anisotropies and emission spectra immediately recorded upon excitation at 360 nm.

As shown by the results included in Fig. 6-5, the emission anisotropy of EDANS-monophosphatase is progressively increased upon addition of increasing concentrations of Hsc 70 to the dilution mixtures. This increase in emission anisotropy is paralleled to a small enhancement of the probe fluorescence. The change in emission anisotropy is caused by the formation of EDANS-monophosphatase / Hsc 70 complex as indicated by


A
EMISSION ANISOTROPY
0.15
0.10
0.05
0
1
2
3
4
5
Hsc 70 uM

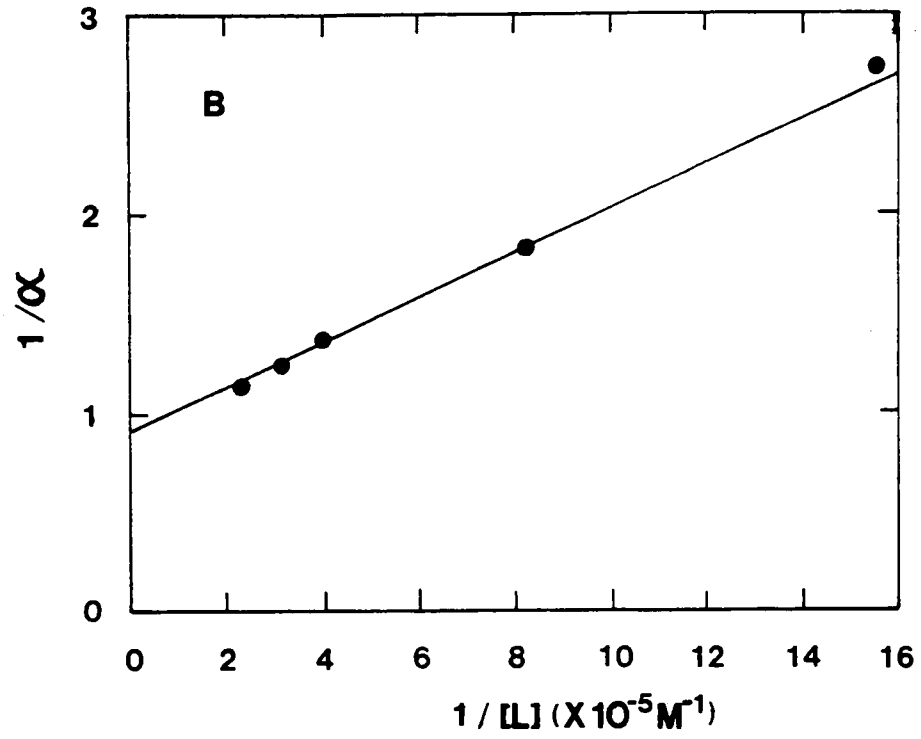



**Fig. 6-5.** *Titration of EDANS-IMPase with Hsc 70.*

**(A)** Changes in the emission anisotropy (A) of EDANS-*myo*-inositol monophosphatase (1 *u*M) on titration with increasing concentration of Hsc 70. Results obtained with samples of the enzyme (20 *u*M) treated with 4M Guanidine HCl for 30 min at 20 °C and diluted 20 fold into the 50 mM Hepes buffer (pH 7.0) containing varied concentration of Hsc 70 protein in the absence (•) and presence of ATP (Δ). Open circles (o) represent results obtained with native EDANS-IMPase without treatment of Guanidine HCl. Experiments conducted at 20 °C with polarized light of 360 nm.

**(B)** Analysis of the titration curves obtained when EDANS-IMPase (1 *u*M) was allowed to interact with Hsc 70. The plot of $1/\alpha$ against $1/[Hsc]_f$, yields a dissociation constant of 1.2 *u*M.

the following reaction;

$$\text{EDANS-E} + \text{Hsc 70} \rightleftharpoons (\text{EDANS-E}) - \text{Hsc 70}$$

In the presence of excess Hsc 70, the equilibrium is shifted in the direction of formation of the protein-protein complex, and the emission anisotropy approaches the maximum value $A_m = 0.15$.

According to the results shown in Fig. 6-5 (A), the concentration of Hsc 70 required to obtain the maximum emission anisotropy was 5 $u$M, whereas the concentration of EDANS-monophosphatase was 1 $u$M. The fraction of EDANS-monophosphatase bound to Hsc 70 was calculated with the aid of equation (4).

$$\alpha = \frac{A - A_f}{(A_m - A)\beta + (A - A_f)} \qquad (4)$$

where A is the observed emission anisotropy, $A_f$ and $A_m$, are the emission anisotropies of free and bound monophosphatase, respectively, and $\beta$, the ratio of fluorescence yields of bound and free EDANS-monophosphatase, is 1.2.

The concentrations of bound and free EDANS-monophosphatase were determined with the aid of equations (5) and (6), respectively.

$$[B] = \alpha \cdot [\text{EDANS-E}]_t \qquad (5)$$

$$[F] = [\text{EDANS-E}]_t - [B] \qquad (6)$$

The results of the titration experiments, fitted to the equation for equivalent binding sites using the enzfitter program of R.J. Leatherbarrow, Biosof, yield a dissociation constant of $K_d = 1.2$ $u$M for EDANS-E complexed to Hsc 70 (Fig. 6-5B, Table 6-1). As expected, dilution of the reaction mixture shifts the equilibrium in the direction of dissociation and the emission anisotropy is decreased. Surprisingly, the values of the emission anisotropy are not perturbed by addition of ATP (Fig. 6-5A), suggesting that the energy released by hydrolysis of ATP does not bring about dissociation of the complex.

The degree of exposure of the probe attached to the monophosphatase to collisional encounters with potassium iodide molecules in the absence and presence of

Hsc 70 was derived from fluorescence quenching measurements. If the probe is buried by the formation of a stable protein-protein complex, then a decrease in the accessibility of the probe to collisional encounters should be observed. This expectation is borne out by the results included in Fig. 6-6, where it can be seen that EDANS-monophosphatase is more exposed to collisional quenching in the absence of Hsc 70.

Recovery of Catalytic Activity of Unfolded Monophosphatase

The effect of Hsc 70 on the recovery of the catalytic activity of unfolded monophosphatase was investigated using the coupled enzymatic assay described in "Experimental Procedures". Samples of EDANS-inositol monophosphatase (26 *u*M), incubated with 4M guanidine HCl in 50 mM Hepes buffer (pH 7) for 30 minutes at 20 °C, were diluted 20 fold with solutions containing 10 uM Hsc 70, 0.1 mM ATP and 0.1 mM $MgCl_2$. After incubation at 20 °C for various lengths of time, aliquots withdrawn from the incubation mixture were used for enzymatic assays.

Samples of unfolded inositol monophosphatase, diluted with buffer alone were run as controls. A typical time course of the reaction monitored at 546 nm is given in Fig. 6-7A. The results of the enzymatic assays are given in Fig. 6-7B, where it can be seen that the recovery of catalytic activity is not influenced by the presence of Hsc 70. It should be emphasized that inositol monophosphatase does not enhance the ATPase activity of Hsc 70 (results not shown).

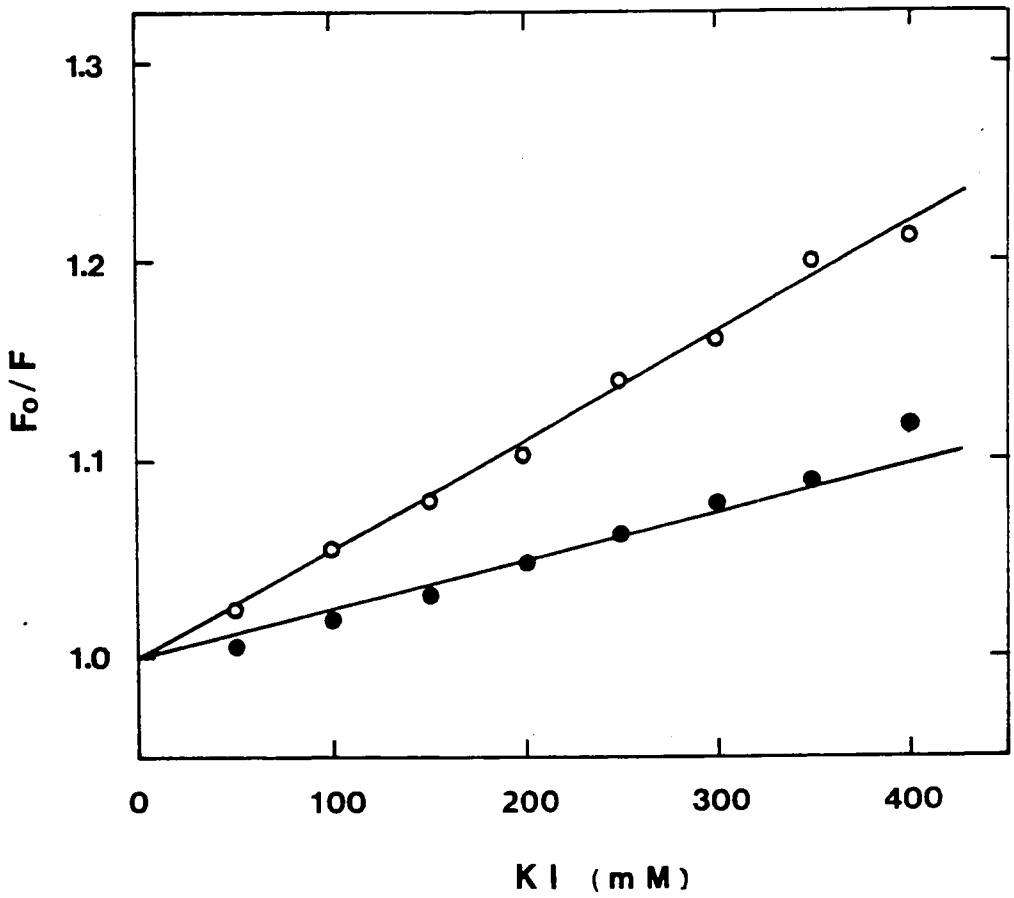
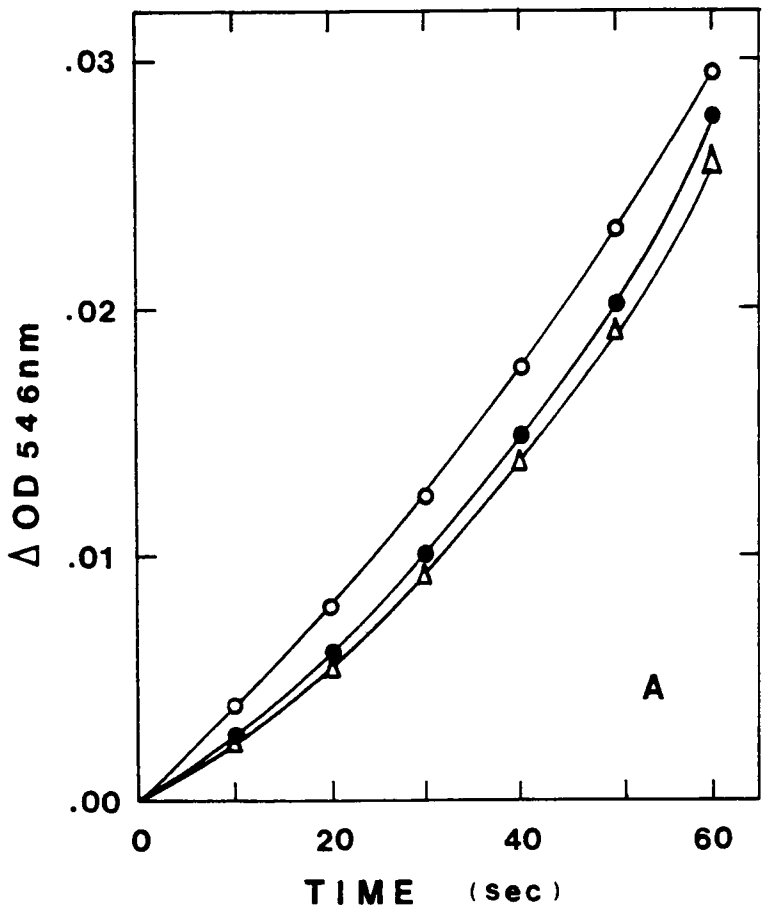



**Fig. 6-6.** *Effect of Hsc 70 on the KI quenching of fluorescence emitted by EDANS-IMPase (1uM).*

Excitation wavelength at 360 nm, emission at 470 nm. Experiments performed at pH 7.0 in the absence (o) and the presence of 3 uM Hsc 70 (•). Solid lines represent the least-square fit with the parameters $K_{sv}$= 0.55 $M^{-1}$(o) and $K_{sv}$=0.25 $M^{-1}$(•).


.03
.02
.01
.00
Δ OD 546nm
0
20
40
60
TIME (sec)
A

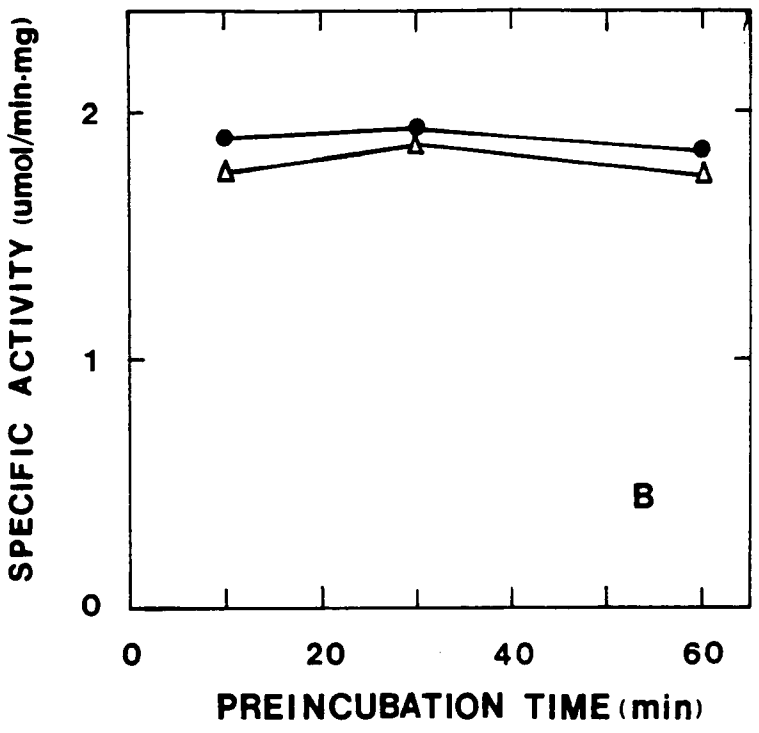



**Fig. 6-7.** *The effect of Hsc 70 on the recovery of the catalytic activity of myo-Inositol monophosphatase.*

(A) The recovery of the catalytic activiy of myo-inositol monophosphatase was monitored by measuring the change in absorbance at 546 nm. IMPase unfolded in 4 M Guanidine HCl was 20 fold diluted into the standard buffer containing 1 mM ATP. This reaction was carried out in the absence (Δ) and presence of Hsc 70 (•) (molar ratio; 1.3 *u*M IMPase : 10*u*M Hsc 70). Aliquots were then withdrawn after incubation for 10 min at room temperature and used for enzymatic assays. The final concentration of *myo*-inositol monophosphatase is 0.026 *u*M in the assay mixture. As a set of control experiments, the sample treated with 4 M Guanidine HCl was directly diluted in the enzymatic assay mixture and assayed for catalytic activity(o).

*Panel* (B) shows the effect of Hsc 70 on the specific activity of the IMPase with longer incubation time. Results were obtained under the same conditions as described in (A), except that longer incubation times were used here.

# DISCUSSION

Fluorescence spectroscopy has been used to gain more information on the interaction of small molecular weight peptides with Hsc 70. Two peptides, an octapeptide corresponding to the amino terminal region of the $\beta$-chain of sickle cell hemoglobin, and a 20 amino acid fragment derived from proteolysis of the amino terminal portion of ribonuclease A, were found to recognize Hsc 70. The observation that an octapeptide containing a high proportion of aliphatic amino acids recognizes Hsc 70 is in agreement with the results of Flynn *et al* (1991), who have reported that the minimum peptide length required for optimal binding is seven amino acids.

On the other hand, the finding that ribonuclease S-peptide also recognizes Hsc 70 is rather surprising since this 20 amino acids peptide contains some elements of secondary structure (1982). This implies that either Hsc 70 recognizes incompletely folded polypeptide chains or that the cluster of 6 hydrophobic amino acids pertaining to the carboxyl terminal portion of the RNase S-peptide is the site of attachment to the protein.

Although it has been reported that binding of ATP to dna K proteins triggers conformational changes detectable by fluorescence spectroscopy (Palleros *et al.*, 1991), we were unable to observe any blue spectral shift in the protein Hsc 70 induced by ATP binding. Indeed, the emission spectra recorded at various excitation wavelengths indicate exposure of the tryptophan residues to a rapidly relaxing aqueous solvent even in the presence of ATP.

It is generally accepted that Hsc 70 binds unfolded conformations of proteins, such as reduced carboxymethylated $\alpha$-lactalbumin (Palleros et al., 1991) and apocytochrome c (Sadis & Hightower, 1992), but it has also been reported in the literature the interaction of Hsc 70 with folded conformations of proteins, *i.e.*, calmodulin

(Stevenson & Calderwood, 1990). Most of these studies have not been concerned with the determination of the dissociation constant of the protein complexes.

An interesting aspect of our work is the finding that the technique of steady-state emission anisotropy can be exploited to obtain information on the dissociation constant of stable protein-Hsc 70 complexes in solution. Using this fluorescence technique, we have found that native inositol monophosphatase, a cytosolic enzyme, does not bind to Hsc 70, whereas the unfolded conformation of the monophosphatase, obtained by treatment with 4 M guanidine HCl, interacts with Hsc 70 with a dissociation constant of 1.2$u$M. The addition of ATP does not influence the stability of the complexes as revealed by measurements of the emission anisotropy of EDANS-E / Hsc 70 complexes in the absence and presence of ATP. The extent of recovery of inositol monophosphatase activity is not influenced by the presence of excess Hsc 70 in the incubation mixtures containing the enzyme and the chaperon protein.

In view of these observations, it is pertinent to ask what is the role of Hsc 70 in the folding of inositol monophosphatase ? It is currently believed that Hsc 70 assists in the correct folding of polypeptide by disentangling aggregated proteins using the energy provided by ATP hydrolysis (Lewis & Pelhman, 1985; Rothman & Kornberg, 1986). Alternatively, binding of Hsc 70 may simply stabilize unfolded conformations of their target polypeptides, preventing the formation of inappropriate intermolecular interactions (Gething & Sambrook, 1992).

In the case of the cytosolic protein inositol monophosphatase complexed to Hsc 70, we were unable to detect any dissociation of the complex facilitated by ATP hydrolysis. Moreover, the weak ATPase activity of Hsc 70 was not enhanced by the protein substrate.

Therefore, we favor the hypothesis that interaction of Hsc 70 with unfolded proteins in the cell may be able to delay the formation of misfolded structures;

particularly when stress conditions, *i.e.*, high temperature and altered redox potential, would perturb the folding pathway of a protein and increase the probability of misfolding.

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# APPENDIX

# STRUCTURAL AND FUNCTIONAL STUDY OF PYRIDOXINE-5'-P OXIDASE

# CATALYTIC AND REGULATORY PROPERTIES OF NATIVE AND CHYMOTRYPSIN TREATED PYRIDOXINE-5'-PHOSPHATE OXIDASE

## INTRODUCTION

Several laboratories have shown that the flavoprotein pyrodoxine-5'-P oxidase, isolated from brain and liver tissues, exhibits product inhibition; the *Ki* for pyridoxal-5'-P ranges from 1 to 5 *u*M depending upon the conditions (pH and buffer) of the enzymatic assays (Kwok & Churchich, 1980; McCormick, 1980). The inhibitory effect exerted by pyridoxal-5'-P may be one of the mechanisms contributing to the regulation of the concentration of cofactor in brain and liver tissues, as originally suggested by Snell and Haskell (Snell & Haskell, 1971b).

Recent studies have demonstrated that modulation of the catalytic activity of pyridoxine-5'-P oxidase can be attained by the reversible formation of a Schiff base between the e-amino group of a specific lysyl residue of the protein and the carbonyl group of pyrodoxal-5'-P (Choi *et al.*, 1987). Regulation of the catalytic activity of liver pyridoxine-5'-P oxidase by tryptophan metabolites has been investigated by Takeuchi *et al.* (1985), who have found that 3-hydroxykynurenine and 3-hydroxyanthranilate activate pyridoxine-5'-P oxidase.

A complete steady-state kinetic analysis of the catalytic parameters influenced by tryptophan metabolites has not been reported, and there is no information available on the mechanism underlying the activation process. It is the main purpose of this work to demonstrate that modulation of the catalytic behavior of brain pyridoxine-5'-P oxidase is achieved by reversible binding of tryptophan metabolites to regulatory sites distinct from the catalytic site.

## EXPERIMENTAL PROCEDURES

### Enzyme Purification

The method used in the purification of sheep brain pyridoxine-5'-P oxidase was based on the method originally applied to the purification of the enzyme from pig brain (6). Enzymatic activity was measured at 25 °C in 0.1 M Tris-HCl (pH 8.3) by monitoring the formation of pyridoxal-5'-P, which has an absorption maximum at 414 nm in Tris. The assay mixture consisted of pyridoxine-5'-P (20 uM) in a total volume of 1 ml.

### Enzymatic Assays

The enzymatic activity of pyridoxine-5'-P oxidase was measured at pH 8.4 in 0.1 M Tris-HCl (25 °C). The rate of formation of pyridoxal-5'-P was measured by following the increase in absorbance at 414 nm. At this wavelength, the Schiff base formed between Tris and pyridoxal-5'-P has an extinction coefficient of 5900 $M^{-1}$ $cm^{-1}$.

A molecular weight of 56,000 for pyridoxine-5'-P oxidase with a specific activity of 0.021 *u*mol $min^{-1}$ $mg^{-1}$ was used in the calculations of molar enzyme concentrations. One unit of specific activity is defined as the amount of protein that catalyzes the formation of 1 *u*mol of pyridoxal-5'-P/min at 25 °C.

Initial rate measurements were carried out by monitoring the change in absorbance at 414 nm for at least 3 min. The initial velocity data were fitted by a least-squres mothod to the Lineweaver-Burk transformation of Equation 1 :

$$v = V[S]/K + [S] \qquad (1)$$

where [S] represents the concentration of the varied substrate pyridoxine-5'-P, and $K$ represents the Michaelis constant. For substrate inhibition, the resulting steady-state rate expression takes the form:

$$v = \frac{V}{1 + (K/[S]) + ([S]/Ki)} \qquad (2)$$

and $K_i$, the inhibition constant, was readily extracted from a plot of 1/v versus [S] at high substrate concentrations.

Chymotryptic Digestion

Pyridoxine-5'-P oxidase (2 mg/ml) was incubated with chymotrypsin (0.1 mg/ml) in 100 mM sodium phosphate (pH 7) at 25 °C for 1 h. Aliquots withdrawn from the incubation mixture at several time intervals were allowed to react with trypsin inhibitor (0.2 mg/ml) and were used for enzymatic assays and SDS-polyacrylamide gel electrophoresis (Laemmli, 1970).

Purification of Partially Digested Oxidase

For ion-exchange chromatography, a TSK DEAE-5PW column fitted to an HPLC instrument (Pharmacia LKB Biotechnology, Inc.) was used for the purification of the peptides obtained after chymotrypsin digestion of pyridoxine-5'-P oxidase.

The peptide applied to the column were eluted using a linear gradient from 0 to 100 % solvent system B (solvent system A = 5 mM sodium phosphate (pH 7.4) ; solvent system B = 0.2 M NaCl in 5 mM sodium phosphate (pH 7.4) at 25 °C.

Fractions collected from the column were monitored at 280 nm, assayed for catalytic activity, and used for determination of FMN content by means of fluorescence spectroscopy in the presence of 2M guanidinium HCl. Excitation was at 450 nm, and emission was at 530 nm.

Binding of 3-Hydroxyanthranilate

Fluorometric titrations at a fixed concentration of enzyme (25 uM) and varying concentrations of 3-hydroxyanthranilate were conducted in 0.1 M sodium phosphate (pH 7.4) using excitation and emission wavelengths of 310 and 420 nm, respectively. fluorescence quenching, elicited by binding of 3-hydroxyanthranilate to the flavoprotein,

was corrected for " inner filter effect", *i.e.* the decrease in fluorescence intensity emitted by 3-hydroxyanthranilate caused by absorption of FMN at 310 and 420 nm.

Two methods were used for correction of inner filter effects : first, Equation (3),

$$F_{Corr} = F_{Obs} \cdot anti\log[(A_{310} + A_{420}) / 2] \qquad (3)$$

where $A_{310} = 0.10$ and $A_{420} = 0.30$ for a solution of the flavoprotein at a concentration of 25 $u$M ; and second, the fluorescence intensity emitted by increasing concentrations of anthranilate in the presence of a fixed concentration of pyridoxine-5'-P oxidase (25 $u$M). Anthranilate, which displays spectroscopic properties identical to 3-hydroxyanthranilate, does not bind to the flavoprotein.

The fluorescence intensities of free ($F_o$) and bound ($F_b$) 3-hydroxyanthranilate as well as the fluorescence corrected when both free and bound ligands are in eqyilibrium ($F$) were used to calculate the fraction of bound effector (Equation 4).

$$\alpha = \frac{F_o - F}{F_o - F_b} \qquad (4)$$

A maximum decrease in the fluorescence emitted by 3-hydroxyanthranilate (80 %) was observed. The average number of molecules of ligand bound per enzyme dimer (56 kDa) was calculated using Equation (5 ):

$$\upsilon = \alpha[L]_o/[P]_o \qquad (5)$$

where $[L]_o$ and $[P]_o$ are total ligand (3-hydroxyanthranilate) and protein concentrations, respectively. The results of the fluorometric titrations were analyzed by plotting $\upsilon/[L]$ *versus* $\upsilon$

Emission spectra were recorded in a spectrofluorometer equipped with two Bausch & Lomb grating monochromators. Absorption spectra were recorded in a Shimadzu UV 160 spectrometer.

Materials

Sephadex G-100 and AH-Sepharose 4B were purchased from Pharmacia LKB Biotechnology Inc. and DE 52 cellulose from Whatman. Protein standards for elctrophoresis experiments were obtained from Bethesda Research Laboratories. Chymotrypsin A and trypsin inhibitor were from Boehringer Mannheim. Pyridoxine-5'-P was prepared by reduction of pyridoxal-5'-P with NaBH4. 3-hydroxyanthranilate and 3-hydroxykynurenine were purchased from Sigma.

## RESULTS

The effects of tryptophan metabolites on the catalytic activity of purified brain pyridoxine-5'-P (PNP) were examined using PNP as a substrate at pH 8.3 (Fig. 7-1, *left*). Substantial substrate inhibition was observed at fixed concentrations of oxygen (0.26 mM) and varying concentrations of PNP. Analysis of the kinetic data as outlined under "Experimental Procedures" yielded an apparent Km value of 15 *u*M and a substrate inhibition constant of 60 *u*M (Table 7-1).

It should be noted that a similar inhibitory effect caused by and accumulation of pyridoxine-5'-P has been observed by Choi *et al.* (1983), who have performed a complete kinetic study of liver pyridoxine-5'-P oxidase. An interesting feature of the activation studies is that the effect exerted by either 3-hydroxyanthranilate or 3-hydroxykynurenine is very pronounced over the PNP concentration range of 15-40 uM when the enzyme is saturated by the substrate. Apparently, the inhibition exerted by accumulation of the substrate PNP at the catalytic site of the oxidase is relieved by the tryptophan metabolites. An increase in the Vmax of pyridoxine-5'-P oxidase is easily detected when the concentrations of 3-hydroxyanthranilate and 3-hydroxykynurenine are 0.03 and 0.3 mM, respectively. The tryptophan metabolites kynurenine, 5-hydroxytryptophan, and anthranilate showed no effect on the catalytic parameters of the oxidase up to concentrations of 5mM at pH 8.3.

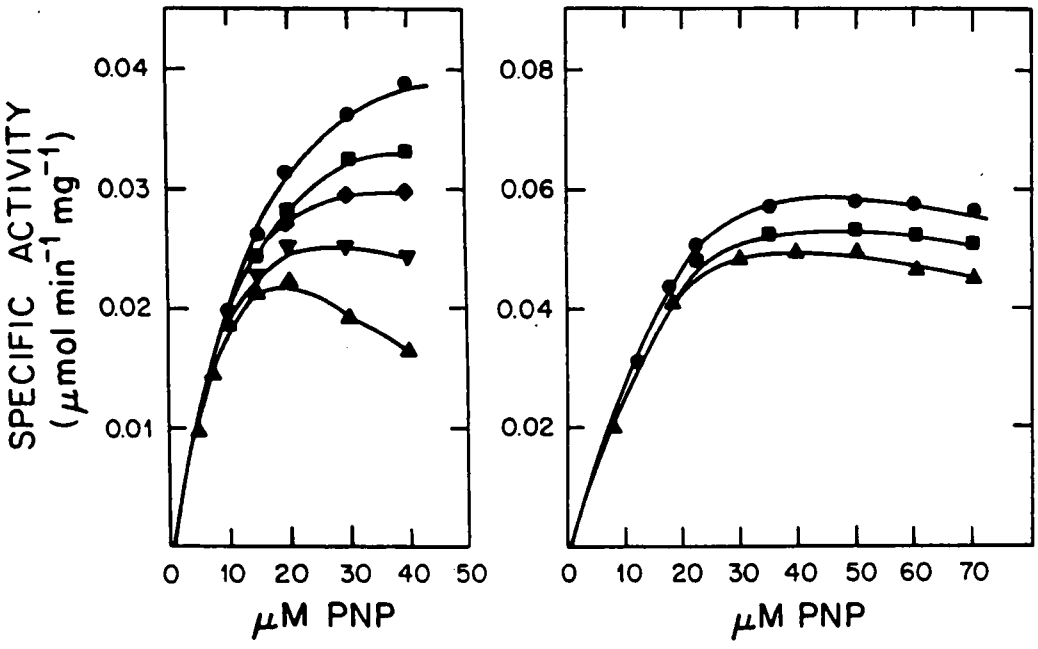


**Fig. 7-1.** *Left, activation of pyridoxine-5'-P oxidase by tryptophan metabolites at 25°C.* Shown is the specific activity of the enzyme plotted versus the concentration of PNP in the absence (▲) and presence of 30 *u*M (◆) and 100 *u*M(•) 3-hydroxyanthranilate and 60 *u*M (▼) and 300 *u*M (■) 3-hydroxykynunenine. The concentration of $O_2$ was kept constant (0.26 mM).

*Right, effect of 100 uM 3-hydroxyanthranilate* (•) *and 300 uM 3-hydroxykynurenine* (■) *on specific activity of pyridoxine-5'-P oxidase treated with chymotrypsin at 25 °C.*

▲, Results obtained in the absence of activators. The concentration of $O_2$ was kept constant (0.26 mM).

**TABLE 7-1**

*Catalytic parameters of chymotrypsin-treated oxidase*

| Sample | Vmax *umol/min/mg* | *Km* *uM* | *Ki*[a] *uM* |
|---|---|---|---|
| Native | 0.022 | 10 | 60 |
| Chymotryp-treated[b] | 0.060 | 15 | - |

Assays were conducted at pH 8.3 in 0.1 M Tris-HCl.

[a] Inhibition constant for the substrate pyridoxine-5'-P

[b] Sample purified by ion-exchange chromatography

Binding of 3-Hydroxyanthranilate

To determine the affinity and stoichiometry of binding of the effectors to the enzyme, fluorometric titrations of pyridoxine-5'-P oxidase were conducted at pH 7.4. 3-hydroxyanthranilate was used as a probe of regulatory binding sites on the enzyme because its spectroscopic properties, *i.e.*, excitation at 310 nm and emission at 420 nm, differ from those of bound FMN. Moreover, free 3-hydroxyanthranilate is fluorescent (quantum yield = 0.20), whereas the emission of bound FMN is completely quenched. When pyridoxine-5'-P oxidase was mixed with a 2 fold molar excess of 3-hydroxyanthranilate at pH 7.4 and the emission spectrum was immediately recorded, it was observed that binding to the enzyme decreased the fluorescent yield of the effector (Fig. 7-2). This quenching effect was used to determine the stoichiometry of binding of 3-hydroxyanthranilate when titrations at a fixed concentration of enzyme were performed as outlined under "Experimental Procedures". Fig. 7-3 depicts a typical plot of $\upsilon/[L]$ *versus* $\upsilon$ obtained from the titration results. The straight lined intercepts the abscissa at $\upsilon = 4$, indicating the presence of four binding sites/enzyme dimer, the recorded differential spectrum of bound FMN did not show any perturbation attributable to the formation of a charge transfer complex (data not shown). The absorption spectra results are taken as an indication that 3-hydroxyanthranilate does not influence the catalytic parameters of PNP oxidase by direct binding to the cofactor FMN.

Chymotrypsin-treated Oxidase

In view of the preceding results, we considered it interesting to investigate whether or not 3-hydroxyanthranilate binds to the structural domain of pyridoxine-5'-P oxidase associated with catalytic activity. Limited chymotryptic cleavage of pyridoxine-5'-P oxidase provides polypeptides endowed with catalytic activity (Kim & Churchich, 1989) ; therefore, limited proteolysis was chosen as the method for the investigation of the presence of functional domains. The enzyme (2 mg/ml) was incubated with

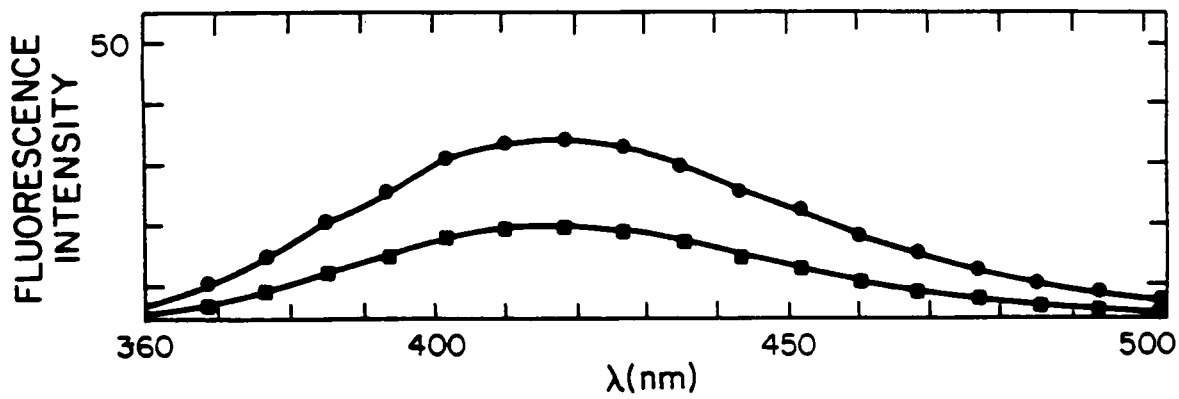


**Fig. 7-2.** *Emission spectra of 3-hydroxyanthranilate* (•) *(50 uM) and 3-hydroxyanthranilate (50 uM) + pyridoxine-5'-P oxidase (25uM)* (■).

Excitation wavelength of 310 nm. The spectra are corrected for inner filter effect as indicated under "Experimental Procedure".

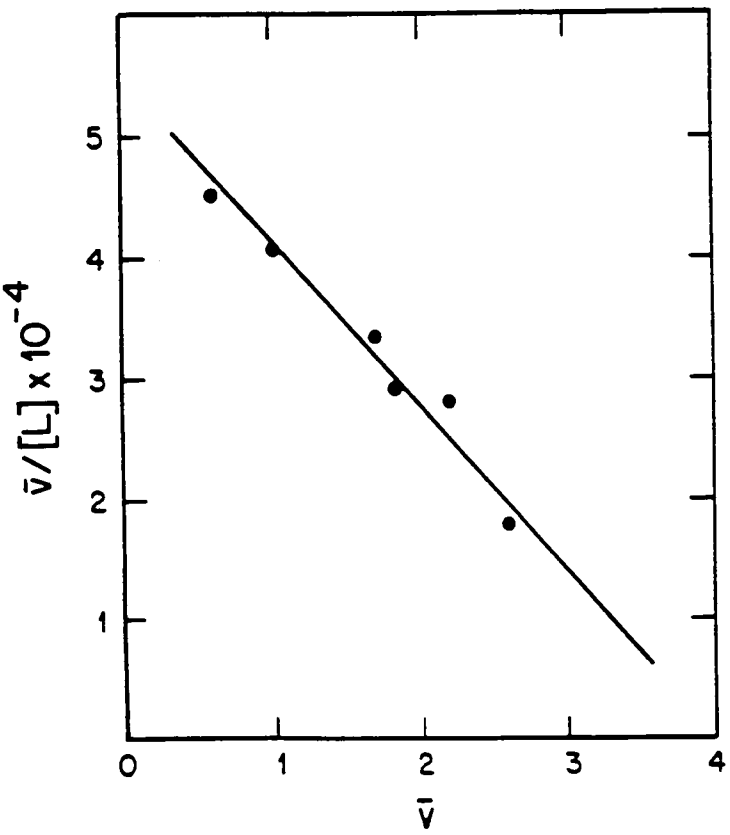


**Fig. 7-3.** *Analysis of titration results conducted at fixed concentration of pyridoxnie-5'-P (25 uM) and varying concentrations of 3-hydroxyanthranilate in 0.1 M sodium phosphate (pH 7.4).*

Shown is a plot of $\upsilon/[L]$ versus $\upsilon$. An association constant value of $K_a = 5.5 \times 10^4\ M^{-1}$ for four binding sites / enzyme dimer was determined at 25 °C.

chymotrypsin (0.1 mg/ml) in 0.1 M sodium phosphate (pH 7) at 25 °C. Aliquots withdrawn from the incubation mixture were used for enzymatic assays and SDS-PAGE.

The overall catalytic activity of pyridoxine-5'-P oxidase increases in the manner depicted in Fig. 7-4 (*left*). After 50 min of incubation at 25 °C, the catalytic activity reached a plateau, whereas the electrophoretic patterns revealed the presence of two major bands of 16 and 12 kDa together with undigested monomeric oxidase (28 kDa) (Fig. 7-4, *right*). It is interesting to note that the SDS-polyacrylamide gel electrophoretic patterns of samples digested for shorter periods of time display two polypeptides as the major products of proteolysis together with undigested oxidase.

Prior to the enzymatic assays of the sample treated with chymotrypsin for 50 min, the proteolytic reaction was stopped by addition of 0.5 mg/ml trypsin inhibitor. The results of enzymatic assays over a wide range of substrate concentrations are given in Fig. 7-1 (*right*), where it can be seen that addition of 3-hydroxyanthranilate (0.1 mM) and 3-hydroxykynurenine (0.3 mM) has a small effect on the Vmax of the enzyme.

The small increase in catalytic activity elicited by 0.1 mM 3-hydroxyanthranilate could be attributed to the presence of ~25 % undigested oxidase in the reaction mixture.

Separation of the polypeptides arising from chymotryptic cleavage of the oxidase was attained by ion-exchange chromatography on a TSK DEAE-5pw column attached to an HPLC instrument. As shown in Fig. 7-5, at least four different fractions absorbing at 280 nm were eluted from the column using a linear gradient from 0 to 100 % solvent system B. One of the elution peaks (retention time of 200 min) contains FMN and displays catalytic activity (Table 7-1). The first fraction eluted from the column exhibits a retention time identical to that of native pyridoxine-5'-P oxidase.

When the fractions endowed with catalytical activity were subjected to a second chromatography and analyzed by SDS-PAGE, we found that only one band of 16 kDa was stained by Coomasie Blue (Fig. 7-6). Based on these results, it is postulated that the structural domain associated with catalytic activity composes approximately one-half of

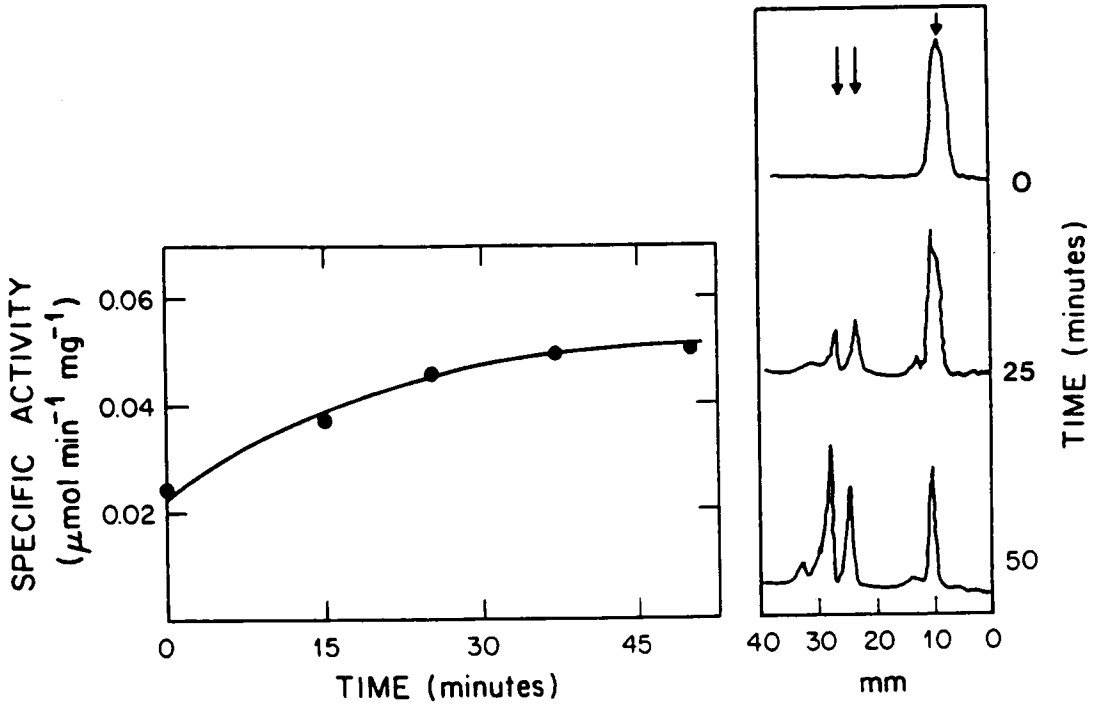


**Fig. 7-4.** *Left, catalytic activity of chymotrypsin-treated pyridoxine-5'-P oxidase.*

Oxidase (2 mg/ml) in 0.1 M sodium phosphate (pH 7) at 25 °C. Aliquots withdrawn from the incubation mixture were used to measure catalytic activity in the presence of pyridoxine-5'-P (20 *u*M) at pH 8.3 in 0.1 M Tris-HCl.

*Right, Pyridoxine-5'-P oxidase (2 mg/ml) incubated with chymotrypsin (20:1, w/w) in 0.1 M sodium phosphate (pH 7.4) at 25 °C (see "Experimental Procedures").* At different times, aliquots were subjected to SDS-PAGE, followed by densitometric analysis of the Coomasie Blue-stained protein bands. Results of the densitometric measurements of sample incubated for 0,15, and 50 min with chymotrypsin are shown. The arrow indicate the band position of monomeric pyridoxine-5'-P oxidase (28 kDa). The following proteins were used as macromolecular markers: lysozyme, trypsin inhibitor, carbonic anhydrase, ovalbumin, bovine serum albumin, and phosphorylase a.

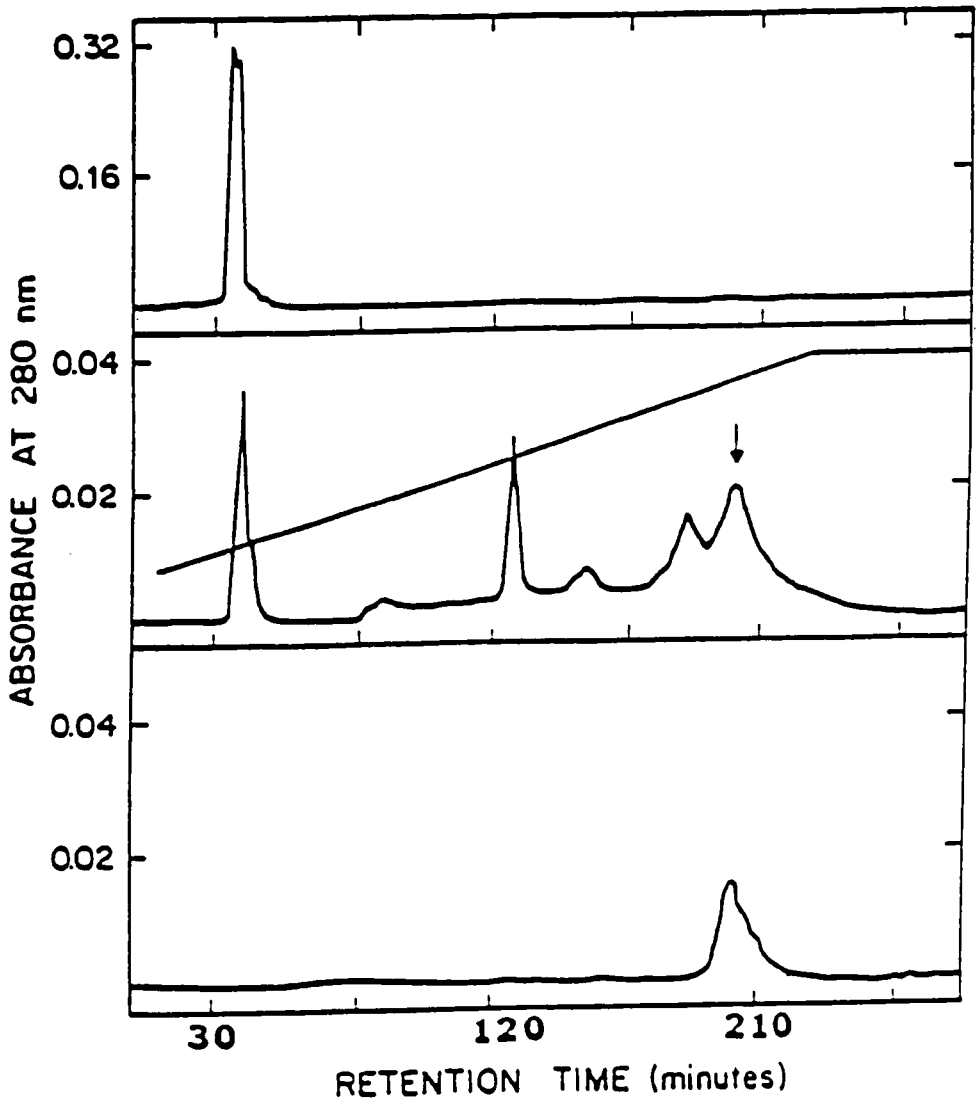


**Fig. 7-5.** *Separation of chymotryptic PNP-oxidase fragments by HPLC.*

0.20 mg of pyridoxine-5'-P oxidase digested with chymotrypsin for 50 min at 25 °C (see "Experimental Procedures") was applied to a TSK DEAE-5pw column and eluted with a linear gradient from 0 to 100 % solvent system B at a flow rate of 0.1 ml/min. The sepatation procedure was repeated three times. Fractions eluted from the column with retention times >190min exhibited catalytic activity. They were pooled, desalted, concentrated, and subjected to a second chromatography (*bottom panel*). The elution profile of native pyridoxine-5'-P oxidase is included (*top pannel*).

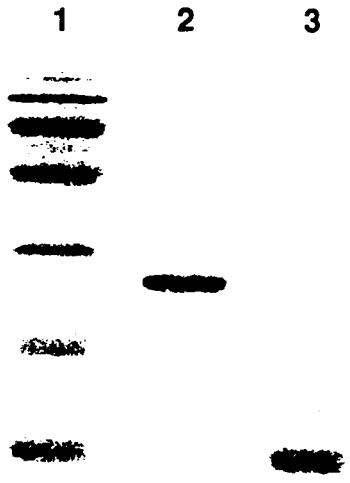


**Fig. 7-6.** *SDS-polyacrylamide gel electrophoresis patterns of standards of known molecular mass (lane 1), native pyridoxine-5'-P oxidase (lane 2), and the polypeptide endowed with catalytic activity (lane 3).*

The standards are : phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme.

the molecular mass of pyridoxine-5'-P oxidase (monomeric). The remaining portion of the macromolecule contains effector binding sites recognized by the tryptophan metabolites 3-hydroxyanthranilate and 4-hydroxykynurenine.

## DISCUSSION

Pyridoxine-5'-P oxidase isolated from brain and liver tissues is a dimeric protein made up of two equal size subunits (the holoenzyme contains one FMN molecule / dimer), but the function of the two subunits in catalysis to be elucidated (Churchich, 1984). The results of steady-state kinetic measurements reported in this work indicate that two tryptophan metabolites, 3-hydroxyanthranilate and 3-hydroxykynurenine, enhance the catalytic activity of the oxidase when the enzyme is saturated with pyridoxine-5'-P in the presence of either tryptophan metabolite. Inhibition induced by accumulation of the substrate pyridoxine-5'-P is completely relieved.

Several lines of experimental evidence indicate that the effector molecules are bound to sites on the protein distinct from the cofactor binding site. Direct determination of the affinity of 3-hydroxyanthranilate for the enzyme by fluorescence spectroscopy has shown the presence of four binding sites/dimer, whereas the dimeric protein contains one FMN binding site. Spectroscopic measurements of the cofactor bound to the enzyme failed to detect any direct interaction between FMN and 3-hydroxyanthranilate (charge transfer complex). Chymotrypsin-treated oxidase, endowed with full catalytic activity, exhibits a small response to the effector 3-hydroxyanthranilate, but negligible response to 3-hydroxykynurenine.

All these results are consistent with the hypothesis that effector molecules capable of modulating the catalytic function of pyridoxine-5'-P oxidase recognize structural domains of the enzyme distict from the catalytic domain.

Although the structure of pyridoxine-5'-P oxidase remains tobe elucidated, it can be envisaged that binding of tryptophan metabolites to the enzyme would have at least one important effect : it would induce conformational changes in the dimeric structure that are transmitted through the subunit interface to the structural domain bearing the cofactor FMN.

The effect of limited proteolysis on the catalytic parameters of flavoproteins has attracted the attention of other laboratories. A stable 37 kDa polypeptide, obtained from limited tryptic digestion of native D-amino acid oxidase, retains full catalytic activity with unaltered kinetic parameters (Tarelli *et al.*, 1990). The terminal 2.0 kDa portion of the protein is not essential for catalysis, but it could play a role in the recognition and import of the enzyme into peroxisomes.

The studies conducted with pyridoxine-5'-P oxidase and D-amino acid oxidase demonstrate that proteolysis is a very powerful tool for the investigation of the function of structural domains in flavoproteins.

In view of the effects exerted by two tryptophan metabolites on the activity of pyridoxine-5'-P oxidase, it is pertinent to ask whether similar effects would be observed under *in vivo* conditions. Tryptophan metabolism is affected by vitamin $B_6$ availability because several of the enzymes participating in this pathway require pyridoxal-5'-P as cofactor. Thus, deficiency of vitamin $B_6$ increases the concentration of some tryptophan metabolites in the cell, *i.e.*, xanthurenic acid, 3-hydroxykynurenine, and 3-hydroxyanthranilate (Donald, 1986; Tryfiates, 1986). Accumulation of tryptophan metabolites in the cell and its interrelationship with the regulation of pyridoxal kinase and pyridoxine-5'-P oxidase are a controversial subject. Conflicting reports on the inhibition of pyridoxal kinase by tryptophan metabolites have been published by two laboratories. Karawya *et al.* (1981) have reported inhibition of liver pyridoxal kinase by xanthurenic acid, 3-hydroxykynurenine, and 3-hydroxyanthranilate ($Ki \sim 1$mM), whereas Takeuchi *et al.* (1985) have reported inhibition byxanthurenic acid, a weak competitive inhibitor with

respect to pyridoxal. Thus, accumulation of xanthurenic acid up to concentrations of 1 mM in a vitamin $B_6$-deficient state may inhibit pyridoxal kinase and decrease the content of pyridoxal-5'-P *in vivo*.

In this study, we have found that concentrations of 3-hydroxykynurenine and 3-hydroxyanthranilate of 0.1 and 0.03 mM, respectively, are sufficient to activate brain pyridoxine-5'-P oxidase.

If, in a vitamin $B_6$ normal state, the metabolites 3-hydroxykynurenine and 3-hydroxyanthranilate approach the concentration level of 0.1 mM, then one would expect specific effects on the function of pyridoxine-5'-P oxidase rather than pyridoxal kinase.

# VITA

Oh-Shin Kwon was born in Kyungju, Korea, on July 8, 1961. He graduated from Simin High School, Taegu, in 1979. He then entered Kyungpook National University, Taegu, in March, 1979 and graduated with a Bachelor of Science degree in Chemistry in February, 1983. He entered the graduate school of the same university and received a Master of Science degree in Bioorganic Chemistry in February, 1985. Upon the graduation, he was drafted into the Korean Army for obligatory military service. After being discharged from the service in 1987, He was an instructor and researcher in the Alma Mater university.

In the fall of 1989, he accepted a graduate assistantship at the University of Tennessee, Knoxville and began his graduate study. During the period of graduate work, he received a Science Alliance upgrade award three times (1991, 1992 and 1993). He received his Ph. D. in Biochemistry in December, 1993. He is currently expecting a post-doctoral position to enable him continue research work in Neuroscience.

He has the following research publications :

**Kwon, O. S.** and Churchich, J. E. (1991) Reaction of pyridoxamine with carboxylic residues of protein. in " *Enzyme Dependent on Pyridoxal Phosphate and other Carbonyl Compounds as Cofactors*" Pergamon Press.

**Kwon, O. S.** , Kwok, F. and Churchich, J. E. (1991) Cataytic and Regulatory of Native and Chymotrypsin treated Pyridoxine-5-Phosphate Oxidase. *J. Biol. Chem.* **266,** 22136-22140.

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Pineda, T., **Kwon, O. S.**, Serpersu, E. H. and Churchich, J. E. (1993) Interaction of ATP analogs with yeast 3-phosphoglycerate kinase, Affinity labeling of the hinge region. *Eur. J. Biochem.* **212**, 719-726

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**Kwon, O. S.** and Churchich, J. E. (1993) Interaction of bovine Hsc 70 with peptides and myo-inositol monophosphatase. *J. Biol. Chem.* (in press).

## 3 Abstracts of Papers

presented at meetings ; 8th International Symposium on Vitamin B6 (1990) in Japan, and ASBMB/DBC-ACS Joint Meeting in Atlanta, GA (1991) and in San Diego, CA (1993) published in *FASEB J.*