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ENZYMATIC STUDIES ON VITAMIN B6 METABOLISM

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비타민은 동물과 어류에 있어 성장, 발육, 및 대사기능에 비교적 적은 양으로 요구되는 필수 미량원소이다. 이들 미세 영양분이 결핍됨으로써 식욕 감퇴에서부터 심한 조직 변형등의 증상들을 일으킨다. 수용성 vitamin B6는 비교적 적은양이 요구되며, 주로 보조 효소 기능을 가지고 있다. 비타민 B6라는 명칭은 유사한 대사 기능들을 가진 화학적으로 관련된 pyridoxine, pyridoxamine, pyridoxal들을 의미한다. 비타민 B6는 비 반추 포유류, 조류와 어류의 음식물을 통해 섭취되는 성분이며, 비타민 B6 화합물들은 식물과 미생물 등에 의해 합성된다.

대사적으로 활성형인 비타민 B6 보조효소는 pyridoxal-5-phsphate(PLP)이며, decarboxylases, aminotransferases, sulfhydrases, tryptophanases를 포함한 아미노산 대사에 관여하는 여러 효소들(PLP-dependant)에 coenzyme으로 작용한다. 비타민 B6 요구량이 육식 동물보다 고단백질을 섭취하는 어류에서 더 높다. B6는 탄수화물과 지질의 대사에도 역시 관여하며 heme과 serotonin의 합성에 필수적이다. 어류에서 결핍증(현상)은 빨리 나타나는데, 이러한 증세로는 신경계 분열, 경련, 유영 부조화, 피부병변, 부종, 안구돌출, 근 긴장성 경련 등이 포함된다.

비타민 B6는 pyridoxal kinase와 pyridoxine(pyridoxamine) oxidase의 촉매 반응에 의해 생체내 활성형인 PLP로 된다. PLP는 PLP-의존성 효소에서 보조 효소로 필수적인 역할을 하는 관계로 이 논문에서는 비타민 B6 생합성 및 대사와 비타민 B6 의존성 효소(aminotransferase)의 특성과 작용기작에 대한 효소학적 연구를 중점으로 이들 enzyme들의 구조와 기능에 대한 최근 연구 동향을 살펴보고자 한다.

Key Words : Vitamin B6, Pyridoxal kinase, pyridoxine oxidase, aminotransferase

Introduction

Vitamins are organic compounds required in the diet in relatively small quantities for growth, health, and function in animals and fish. Although the requirements are small, deficiencies of these micronutrients can cause symptoms ranging from poor appetite to severe tissue deformities. Deficiency signs indentified in channel catfish, salmonids, common carp, and eel. Water soluble B6 vitamins are required in relatively small quantities and have primarily coenzyme functions. The name, vitamin B6 was given to theree chemically related compounds(Fig. 1) that have similar metabolic functions ; pyridoxine, pyridoxamine, and pyridoxal(2). Vitamin B6 is a dietary requirement of all nonruminant mammals, birds, and fish. Vitamin B 6 compounds are synthesized by plants and some microorganisms.

The metabolically active B6 coenzyme is pyridoxal-5-phosphate(PLP) (1). It is functional in number of enzymes(PLP-dependent enzymes) in which amino acids are metabolized, including decarboxylases, ami-

notransferases, sulfhydrases, tryptophanase, and hydroxylases. Vitamin B6 requirement is higher for fish are fed much higher protein diet than land animals. B6 is also involved in metabolism of carbohydrates and lipids. It is essential for the synthesis of heme and serotonin. Deficiency signs in fish develop quickly, including nervous disorders, convulsions, poor swimming coordination, skin lesions, edema, exophthalmos, and tetany. The requirement for fish is 3 mg /kg to 10mg/kg of diet.

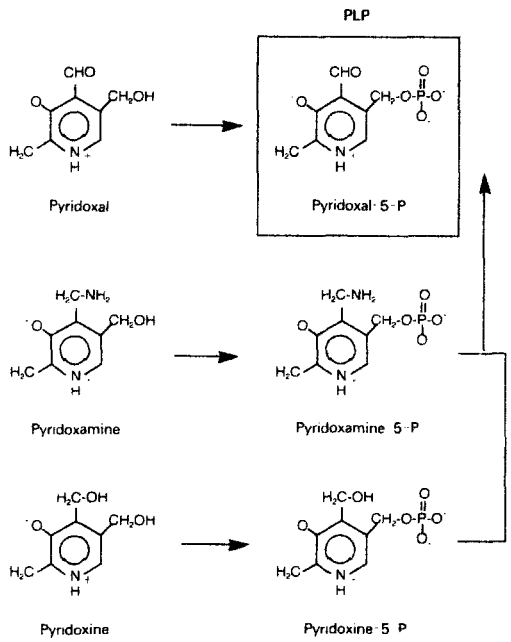

Fig. 1. Bioconversion of vitamin B6.

B6 precursors(pyrodoxine, pyridoxamine, and pyridoxal) are converted to metabolically active form of Vitamin B6, pyridoxal-5-phosphate(PLP). Pyridoxal kinase does main function of the phosphorylation of B6 precursors, whereas pyroxine (pyridoxamine)-5-phosphate are converted to PLP by catalytic activity of the pyroxine(pyridoxamine)-5-phosphate oxidase.

The conversion of vitamin B6 to metabolically active form, pyridoxal-5-phosphate(PLP), is catalyzed by pyridoxal kinase and pridoxine(pyridoxamine) oxidase. PLP serves essential roles in PLP-dependent enzymes as a cofactor(Fig. 2). In this review, we present a brief retrospective view of enzymatic studies on vitamin B6 metabolism(pyridoxal kinase and pyridoxine-5-P oxidase) and about the mechanisms and properties of a vitamin B6 dependent enzyme(aminotransferase).

Pyridoxal kinase

Pyridoxal kinase(pyridoxal kinase(ATP : pyridoxal 5'-phosphotransferase, E. C. 2. 7. 1.35)) is an enzyme of utmost biological interest since it catalyzes the formation of the active species of vitamin B6 from its vitamers. The formation of pyridoxal-P catalyzed by pyridoxal kinase from pyridoxal and ATP is essential for many transamination, decarboxylation and racemization reactions. Besides the conversion of pyridoxal to pyridoxal-P, this enzyme can also phosphorylate pyridoxine and pyridoxamine from ATP(Fig. 1). The phosphorylation of three vitamers by pyridoxal kinase requires the presence of a divalent cation and a monovalent cation. The most effective divalent cation is Zn^{++} and the order of activation is $Zn^{++}>Co^{++}>Mn^{++}>Mg^{++}>Fe^{++}$. The monovalent cation is K^{+}.

In 1944, Gunsalus et al.(1) discovered that the active form of vitamin B6 is pyridoxal-P, which is now known to serve as cofactor in the enzymatic transformations of all amino acids(2). Pyridoxal kinase is the key enzyme responsible for the phosphorylation of the vitamin B6 and has been detected in brain(2), liver (3), *Streptococcus faecalis*(1), *Escherichia coli*(4) and yeast(4). A detailed investigation on the distribution

of pyridoxal kinase in rat and bovine tissues has been conducted by McCormick et al.(5) and brain has been found to be a rich source of the enzyme. Many pyridoxal analogs have been reported to be inhibitors of pyridoxal kinase and these analogs are primarily oximes, azines and hydrazines of pyridoxal. Biogenic amines have also been reported to inhibit pyridoxal kinase(6).

Pyridoxal kinase has also been reported as a key enzyme related to the transport of vitamin B6 in ***Salmonella Typhimurium*** LT2 cells(7). The uptake of pyridoxine by facilitated diffusion through the cell membrane is followed by trapping by pyridoxal kinase. Other studies of transport of vitamin B6 concerning pyridoxal kinase in the brain have been reported (8).

The physiological significance of pyridoxal kinase is not fully known. However, during a comparative study of pyridoxal kinases from different organisms (5), brain tissue has been found to be the richest source of this enzyme. This finding is of particular interest because of the intimate relationship of vitamin B6 to brain metabolism as evidenced by the occurrence of convulsive seizures during vitamin B6 deficiency, whether induced by restricted intake of the vitamin, by administration of analogs of the vitamin or by administration of carbonyl reagents such as isonicotinyl hydrazide and related compounds. Other ***in vivo*** studies in rabbits have demonstrated that when the concentrations of various biogenic amines were increased by compounds which inhibit their metabolism, the activity of pyridoxal kinase in the brain was reduced. Furthermore, chronic treatment with levodopa alters the activity of pyridoxal kinase in rat brain and also elevates the concentration of pyridoxal-P in plasma of Parkinsonian patients.

Procedures for the purification of pyridoxal kinase from bovine, porcine, and sheep brains have been developed(9). Recently, the enzyme isolated from sheep brain has been identified to be a dimer with a molecular weight of 80 kDa(9), and dissociation of the dimeric structure under mild denaturing conditions demonstrated that the monomeric species were catalytically competent(10).

Similar to other well characterized kinases(11, 12), it seems likely that pyridoxal kinase contains a cleft that divides the monomeric protein into two lobes. Some support for this contention was derived from electron microscopy(9) and X-ray crystallographic studies(13).

Previous physical studies have suggested that substrate binding sites of pyridoxal kinase are hydrophobic (14). A 21 amino acid residue polypeptide of the kinase, derivatized with adenosine tetraphosphate pyridoxal(AP_4-PL), was isolated(15). The affinity label reagent(AP_4-PL) has reacted with a specific lysyl residue of monomeric pyridoxal kinase. The peptide was shown to contain 15 hydrophobic amino acids out of a total of 21 residues. Another report(16) showed that a tyrosine residue at the pyridoxal binding site is essential for catalytic activity. However, the exact conformation of this peptide is still unknown, and further X-ray diffraction studies on the enzyme are required for the elucidation of its structure/function relationships.

In a recent report by Kim et al.(17), physical interactions between pyridoxal kinase and aspartate aminotransferase were detected by means of emission anisotropy and affinity chromatography techniques (17, 18, 19). Binding of aspartate aminotransferase (apoenzymes) to pyridoxal kinase tagged with a fluorescent probe(FIT ; fluoresceine isothiocyanate) was

detected by emission anisotropy measurements. The stable protein complex, aspartate aminotransferase (apoenzymes) to pyridoxal kinase, is characterized by a dissociation constant of 3 μM(17). Affinity chromatography also showed the direct protein-protein complex between pyridoxal kinase and PLP-dependent enzyme(17).

A test of compartmentation of pyridoxal-5-phosphate within the protein complex using alkaline phosphatase as trapping agent, indicates that the cofactor generated by the catalytic action of the kinase is channeled to the apotransaminase. The main function of the stable complex formed by the kinase and the aminotransferase is to hinder the release of free pyridoxal-5-phosphate into the bulk solvent(17).

Chymotrypsin digestion of sheep brain pyridoxal kinase, a dimer of identical subunits each of 40 kDa, yields two fragments of 24 and 16 kDa with concomitant loss of catalytic activity. The same pattern of digestion was observed when IAF pyridoxal kinase, carrying a fluorescent probe covalently bound to a specific SH residue, was preincubated with chymotrypsin (20). The kinetics of proteolysis of IAF-pyridoxal kinase was monitored by emission anisotropy ; and the analysis of the initial rate of proteolysis at various concentrations of chymotrypsin reveals that the rate of unfolding of native pyridoxal kinase plays an dominant role in the proteolytic process(20).

Pyridoxine-5′-phosphate oxidase

Pyridoxine(pyridoxamine) 5′-phosphate oxidase(E. C. 1.4.3.5.), a dimeric enzyme with two identical subunits, has molecular weights ranging between 54 to 60 kDa. The enzyme binds FMN at a ratio of 1 mol FMN/ℓ mol dimer. After binding to the apoenzyme, the absorption bands of FMN are shifted from 327nm and 445nm maximum to 380nm and 448nm, respectively, and the ratio of A_{380} : A_{448} for the holoenzyme is significantly higher than the ratio of A_{372} : A_{445} for FMN. This phenomenon has been observed in both the liver and the brain enzyme.

Pyridoxal 5′-phosphate, the metabolically active form of vitamin B6, is the coenzyme required by numerous enzymes involved in transamination, and racemization reactions. Two reaction steps are included in the conversion of pyridoxine and pyridoxamine to pyridoxal 5′-P : (a) phosphorylation catalyzed by pyridoxal kinase and (b) oxidation of the phosphorylated vitamins catalyzed by the FMN-dependent pyridoxine(pyridoxamine) 5′-P oxidase(Fig. 1). The reactions are shown as in the following ;

(a) Pyridoxine / Pyridoxamine + ATP —(Pyridoxal kinase, Zn^{++})→ Pyridoxine 5′-P / Pyridoxamine 5′-P + ADP

(b) Pyridoxine 5′-P + O_2 —(Pyridoxine 5′-P oxidase)→ Pyridoxal 5′-P + H_2O_2

Pyridoxamine 5′-P + O_2 + H_2O —(Pyridoxamine 5′-P oxidase)→ Pyridoxal 5′-P + H_2O_2 + NH_2

Pyridoxine(pyridoxamine) 5′-phosphate oxidase was first found in rabbit liver by Pogell(21) and later studied by many other laboratories including the laboratory of Wada and Snell(22). The localization of this enzyme in mammals covers a wide range of tissues including liver, skeletal muscle, pancreas and bone marrow with relatively lower activities(23). These differences in oxidase activities among different tissues lead 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 exte-

rnal supply to other tissues with low oxidase activities. An example of the distribution netword suggested by Lumeng et al.(24) is that the synthesis of pyridoxal 5′-P in muscle is not adequate for its own tissue consumption and as a result, additional supply of pyridoxal 5′-P has to come from either the liver cells or erythrocytes via the circulation. Each tissue maintains an independent pool of vitamin B6 which includes pyridoxine, pyridoxamine and pyridoxal. The content of different chemical forms of vitamin B6 in this pool is regulated by a combination of different chemical forms of vitamin B6 in this pool is regulated by a combination of enzymes, such as pyridoxine 5′-P oxidase, pyridoxal kinase, different species of phosphatases and various pyridoxal 5′-P binding proteins. Then, the activity of any one of the above enzymes or proteins is also regulated by metabolites from other metabolic pathways. This is demonstrated by findings such as decreases in pyridoxine 5′-P oxidase activity as a result of riboflavin deficiency(25) and the activation of rat liver oxidase activity by 3-hydroxykynurenine and 3-hydroxyanthranilate both of which are metabolites of tryptophan metabolism(26).

Apart from mammalian tissues, pyridoxine 5′-P oxidase activities have also been found in other eukaryotic systems, including yeast(27) and wheat seedlings (28).

In the recent studies by Kim and Churchich(29), chymotryptic digestion of brain pyridoxine-5-P oxidase brings about a 4-fold enhancement of the catalytic power(V_{max} and K_m)using pyridoxine-5-P as substrate in the assay mixtures. The chymotrypsin treated enzyme is less susceptible to inhibition by pyridoxal-5-P than the native enzyme(29).

Fragments arising from limited proteolysis were separated by affinity chromatography using P-pyridoxal-Sepharose as supporting matrix. Catalytically active fractions, eluted by pyridoxine-5-P(5mM), displayed three bands when analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The molecular masses of the three protein bands are considerably lower than 28 kDa, the molecular mass of monomeric pyridoxine-5-P oxidase(30). Spectroscopic studies, absorption, fluorescence, and circular dichroism revealed that the microenvironment surrounding the cofactor flavin mononucleotide is not perturbed by limited proteolysis (29, 30).

4-aminobutyrate Aminotransferase

Metabolically active PLP synthesized by pyridoxal kinase and pyridoxine 5′-P oxidase is utilized by numerous PLP(vitamin B_6)-dependent enzymes as a cofactor. 4-aminobutyrate aminotransferase is one of PLP requiring enzymes. Here, we review structural and functional roles of this enzyme to understand the protein chemistry of PLP metabolic enzyme as an example. 4-aminobutyric acid(GABA) is widely recognized as a major inhibitory neuro-transmitter in many invertebrate systems or in the vertebrate central nerveous system(31) and serves as an important constituent in the intermediary metabolism of brain(32). Upon release from certain nerve terminals, the amino acid activates specific $GABA_A$ receptor on the postsynaptic membrane(33), engendering the opening of Cl^- channels in the membrane. The subsequent hyperpolrization of the postsynaptic nerve cell is the basis of GABA's inhibitory action(34).

The cessation of GABA neurotransmission occurs with the rapid removal of the transmitter from the synaptic gap by means of a high affinity, Na^+- and

Cl^--dependent GABA pump(35, 36). Once taken up, GABA migrates to the mitochondria in preparation for its metabolic degradation by the action of an enzyme complex situated in the matrix(37). This macromolecular unit consists of 4-aminobutyrate aminotransferase(GABAT ; E. C. 2. 6. 1. 19) and succinic semialdehyde dehydrogenase(SSADH ; E. C. 1. 2. 1. 24). The mitochondrial enzyme 4-aminobutyrate aminotransferase(GABAT) is typical pyridoxal-5'-phosphate(PLP) dependent transaminase that catalyzes the transamination of 4-aminobutyrate with the enzyme bound cofactor pyridoxal-5-P to yield succinic semialdehyde and the pyrkdoxamine form of the cofactor. The oxidized cofactor is reformed by transamination with 2-oxoglutarate to yield glutamate and pyridoxal-5-P.

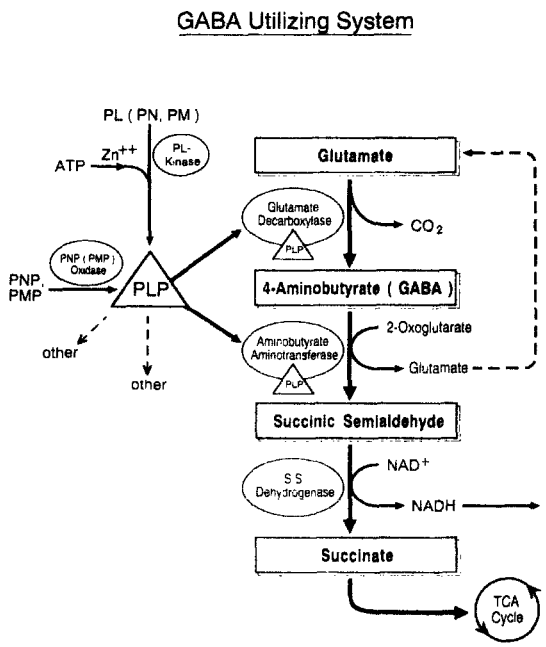

Fig. 2. GABA utilizing system.

The metabolically active PLP is catalyzed by pyridoxal kinase[PK] and pridoxine[PNP](pyridoxamine ; [PMP]) oxidase. It does function as a coenzyme in number of enzymes(PLP-dependent enzymes) in which amino acids are metabolized, including decarboxylases, aminotransferases, sulfhydrases, tryptophanase, and hydroxylases. In the GABA utilizing system, PLP produced by PK or PNP(PMP) oxidase serves as a cofactor of glutamate decarboxylase and aminotransferase. Once the cessation of GABA neurotransmission taken up, GABA migrates to the mitochondria in preparation for its metabolic degradation. The catabolism of GABA results in its entry into the TCA cycle as succinate following transamination with 2-oxoglutarate catalyzed by GABAT. The reaction product, succinic semialdehyde, is subsequently oxidized by succinic semialdehyde dehydrogenase, there by returning the carbon atoms of GABA to the TCA cycle.

The catabolism of GABA results in its entry into the TCA cycle as succinate following transamination with 2-oxoglutarate catalyzed by GABAT(Fig. 2). The quantitative contribution of GABA shunt to glucose oxidation in brain has been estimated to be 10% of the total flux through the TCA cycle(38). Since abnormal levels of 4-aminobutyrate(GABA) in brain have been associated with a variety of neurological disorders including Huntington's disease(39), epilepsy(40), and Parkinsonism(41), it is important to know the structural and catalytic behavior of the enzymes involved in the biosynthesis and degradation of 4-aminobutyrate as well as their regulating mechanisms.

GABAT has been purified to homogeneity from the brain tissues of different mammals including rat, mouse, pig, rabbit, and human. The mammalian aminotransferase has been found to have a molecular weight of approximately 100,000, to consist of two equal molecular weight subunits, and to contain 1 mol

of PLP/enzyme dimer, although there is considerable variability found for the kinetic parameters(i. e. : Km, Vmax).

Although the enzyme has been purified several years ago, little work has been done on its structure. The amino acid sequence of the P-pyridoxal peptide pertaining to the catalytic site of 4-aminobutyrate aminotransferase has been determined(42, 43). This peptide which contains 13 different amino acids, does not contain any cysteine residues. However, it was reported that two sulfhydryl groups of the enzyme are critically connected with the catalytic activity(43). Thus, the blocking of less than 2-SH groups/enzyme dimer by reagent DTNB(5, 5'-dithiobis 2-nitrobenzoic acid : sulfhydryl attacking aregent) abrogates catalytic activity. The reaction of 1.9 SH residues/dimer with iodosobenzoate resulted in enzyme inactivation together with the formation of oligomeric species of M. W. =100,000. The cross-linked subunits are dissociated by addition of β-mercaptoethanol or dithiothreitol which also restores the catalytic activity of iodosobenzoate-inhibited enzyme. Altogether, these observations are consistent with the concept that inactivation of 4-aminobutyrate aminotransferase by iodosobenzoate proceeds through disulfide bond formation between vicinal cysteinyl residues in the dimeric protein ; i. e., they are able to participate in the formation of one intersubunit disulfide bond because they are in close proximity, within 2 Å of each other(43). An important corollary of these studies is related to the position of those critical-SH residues in the spatial structure of the dimeric enzyme. They are situated on opposite sides of the enzyme dimer at the subunit interfaces. Three cysteinyl containing tryptic peptides were isolated and sequenced from mitochondrial 4-aminobutyrate aminotransferase using DABIA(4-dimethylaminoazobenzene-4-iodoacetamide) as specific labeling reagent for sulfhydryl groups. The enzyme is a dimer made up of two identical subunits, but four out of the six cysteinyl residues/dimer form disulfide bonds when treated with iodosobenzoate to yield incative enzyme species. The sulfhydryl groups undergoing oxidation/reduction were found to be intersubunit, based on SDS/polyacrylamide gel electrophoresis results. The loss of catalytic activity of 4-aminobutyrate aminotransferase by oxidation of sulfhydryl residues is related to constraints imposed at the subunit interface by the insertion of disulfide bonds(43).

The microenvironment surrounding the molecule of cofactor tightly bound to the native enzyme has been explored with the aid of ^{31}P-NMR spectroscopy(44). ^{31}P-NMR measurements have shown 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.

4-aminobutyrate aminotransferase is inactivated by preincubation with a PLP analog, P^{1}P^5-bis(5-pyridoxal) diphosphate(bis-PLP). The amino acid sequence associated with bis-PLP binding site and tryptophan containing tryptic peptides have been determined(45).

cDNA clones encoding 4-aminobutyrate aminotransferase have been isolated by using antibodies directed against mature aminotransferase to screen a bovine brain λgt11 DNA library(46) and sequenced by dideoxy chain termination method. Molecular biological approaches are on progress to define the role of specific amino acids in the reaction mechanism and the structural, functional aspects of 4-aminobutyrate aminotransferase.

Concluding remarks

This work summarizes the enzymatic studies on the structural and functional roles of the enzymes : pyridoxal kinase, and pyridoxine(pyridoxamine) 5'-phosphate oxidase, 4-aminobutyrate aminotransferase,.

Physical interactions between pyridoxal kinase and aspartate aminotransferase were detected by means of emission anisotropy and affinity chromatography techniques. A test of compartmentation of pyridoxal-5-phosphate indicates that the cofactor generated by the catalytic action of the kinase is channeled to the apotransaminase. The main function of the stable complex formed by the kinase and the aminotransferase is to hinder the release of free pyridoxal-5-phosphate into the bulk solvent. The kinetics of proteolysis of IAF-pyridoxal kinase was monitored by emission anisotropy ; and the analysis of the initial rate of proteolysis at various concentrations of chymotrypsin reveals that the rate of unfolding of native pyridoxal kinase plays an dominant role in the proteolytic process.

Three cysteinyl containing tryptic peptides were isolated and sequenced from mitochondrial 4-aminobutyrate aminotransferase using DABIA as specific labeling reagent for sulfhydryl groups. The sulfhydryl groups undergoing oxidation/reduction were found to be intersubunit, based on SDS-PAGE. 4-aminobutyrate aminotransferase is inactivated by preincubation with P^1P^2-bis(5-pyridoxal) diphosphate(bis-PLP) at pH 7.0. The amino acid sequence associated with bis-PLP binding site and tryptophan containing tryptic peptides have been determined.

Molecular cloning of 4-aminobutyrate aminotransferase was performed using antibodies directed against mature aminotransferase to screen a bovine brain λgt 11 DNA expression libraty. The cDNA clone coding for 4-aminobutyrate aminotransferase shows the enzymatic activity identical to native enzyme, which catalyzes the conversion of 4-aminobutyrate to succinic semialdehyde.

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Enzymatic Studies on Vitamin B6 Metabolism

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Vitamin B6(pyridoxine, pyridoxamine, and pyridoxal) is a dietary requirement in relatively small quantities for growth, health, and function in animals and fish. The metabolically active B6 is pyridoxal-5-phosphate(PLP). It does function as a coenzyme in number of enzymes(PLP-dependent enzymes) in which amino acids are metabolized, including decarboxylases, aminotransferases, sulfhydrases, tryptophanase, and hydroxylases. Vitamin B6 requirement is higher for fish because fish are fed much higher protein diet than land animals. B6 is also involved in metabolism of carbohydrates and lipids and essential for the synthesis of heme and serotonin. Deficiency signs in fish develop quickly, including nervous disorders, convulsions, poor swimming coordination, skin lesions, edema, exophthalmos, and tetany. The conversion of vitamin B6 to metabolically active form(PLP) is catalyzed by pyridoxal kinase and pridoxine(pyridoxamine) oxidase. In this review, we summarized in detail the enzymatic studies on vitamin B6 metabolism and about the mechanisms and properties of a PLP-dependent enzyme.