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BINDING OF NEW PLP ANALOGS TO THE CATALYTIC DOMAIN OF GABA TRANSAMINASE

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The binding site of Pyridoxal-5-P in 4-aminobutyrate aminotransferase was studied by using analogs of the cofactor. A phosphorothioate analog (PLP(S)) recognizes the catalytic site; it forms a stable complex with the apoenzyme (K_D = 1nM) and serves as cofactor during catalysis. Replacement of a non-bridged oxygen by sulfur in the phosphate side chain renders a compound which preserves the negative charges needed for correct alignment of the cofactor at the catalytic site. This phosphorothioate analog of PLP can be used to investigate the catalytic site of vitamin B_6 dependent enzymes by means of ^{31}P NMR spectroscopy.

A bulky P-pyridoxamine derivative, ie, N-4-azido-2-nitrophenyl pyridoxyl-5-P (NANP) competes with the natural cofactor for its binding site. Upon illumination, the arylazide of P-pyridoxamine acts as an efficient photolabeling reagent of the protein. A characteristic feature of this photolabeling reagent, ie, its ability to recognize the cofactor binding site, can be exploited to ascertain the chemical nature of amino acid residues at the catalytic site.

1. INTRODUCTION

4-aminobutyrate aminotransferase catalyzes the reversible transamination of the neurotransmitter 4-aminobutyrate with the active site pyridoxal-5-P to yield succinic semialdehyde and the pyriodoxamine-5-P form of the enzyme. Pyridoxal-5-P is reformed by transamination with α-ketoglutarate to yield glutamate and enzyme bound pyridoxal-5-P.

The cofactor of the enzyme is firmly bound to a lysyl residue through a Schiff's base linkage, and the amino acid sequence of the peptide carrying the P-pyridoxyl group has been established (1). However, no information is available on the conformation of the catalytic site; and little is know about the chemical nature of the amino acid residues critically connected with catalysis.

A profitable way to investigate the catalytic domain of vitamin B6 requiring enzymes consists in the use of analogs of the coenzyme which are able to interact with amino acids residues located near the cofactor binding site (2,3).

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It is the main purpose of this work to report the results obtained when two new P-pyridoxal derivatives were used as probes of the catalytic site domain of 4-Aminobutyrate Aminotransferase. A phosphorothioate analog of Pyridoxal-5-P is used to probe the interaction of the phosphate side chain of the cofactor with positively charged amino acid residues of the enzyme, whereas a photolabeling reagent N-4-Azido-2-Nitrophenyl-Pyridoxyl-5-P (NANP) is used to modify amino acid residues at the catalytic site.

2. MATERIALS AND METHODS

Synthesis of N-4-Azido-2-Nitrophenyl-Pyridoxyl-5-P 4-fluoro-3-nitrophenyl-azide, (1000, μmol) in 7 ml of ethanol is added to a solution of 600 μmol of pyridoxamine-5-P in 5 ml of water containing 10 mmol of sodium carbonate. Ethanol (14 ml) and another portion of water (6 ml) were added subsequently to enhance the homogeneity of the reaction mixture. The reaction was allowed to proceed at 50° C for 10 hours with an attached cooling condenser. At the end of the reaction period, the resulting dark red mixture was concentrated under vacuum to about 10 ml and then diluted with 10 ml of water. Two extractions with 50 ml of ether removed all of the excess starting azide.

The aqueous solution was concentrated to a volume of 5 ml, acidified with acetic acid to a pH of about 3.2, and applied to a cation exchange resin (Bio-Rad-AG-50W-X4) on a column of 35 x 2 cm. The column was washed with 10 mM NH4-formate (pH 3.2), 100 mM NH4-formate (pH 3.2) and NH4-acetate (pH 4.2) to elute pyridoxamine-5-P. A red compound (N-4-azido-2-nitrophenyl-pyridoxyl-5-P) is eluted with 100 mM NH4-acetate (pH 5.4). All the operations, synthesis and ion exchange chromatography are performed in the dark to prevent photodecomposition. The red compound was chromatographed on cellulose thin layer developed in water/acetone/ter-amylalcohol/acetic acid (20/35/40/5,v/v). One red color spot was detected in this solvent system. The spot turns blue on testing with dichloroquinone-chlorimide. The absorption spectrum of N-4-azido-2-nitrophenyl-pyridoxyl-5-P shows two intense absorption bands centered at 340 and 460 nm, respectively. The infrared spectrum shows a band at 2130 cm-1 which is characteristic of N3 stretching vibrations. Upon

irradiation with light at wavelengths longer than 360 nm, the absorption band at 460nm of N-4-azido-2-nitrophenyl-pyridoxyl-5-P is progressively decreased.

2.1. Synthesis Of Phosphorothioate - PLP.

Pyriodoxal Kinase (1 mg) was incubated with pyridoxal (0.1 mM), ATP (γ) S (0.1 mM) and Cobaltacetate (0.1 mM) in 10 ml of 0.1 M KCl at pH 6.2. The reaction was allowed to proceed at 37° C in the dark for 12 hours. The reaction mixture was concentrated to a final volume of 2 ml and applied to a column (15 x 1 cm) of DEAE Cellulose (DE-52 Whatman) equilibrated with 0.1 M ammonium Acetate (pH4).

The column was eluted with the same buffer, and fractions collected adjusted to pH 7 with NH_4OH prior to spectrophotometric measurements. Fractions absorbing at 390 nm were combined and concentrated in a Liophylizer. The purity of the compound was tested by thin layer chromatography, absorption and P31NMR spectroscopy.

The absortion spectrum of PLP (S) shows an intense absorption band centered at 389 nm (ε = 4.6 x 10^3), and it gives a phosphorus resonance signal which is shifted (21.8 ppm) when compared to phosphoric acid. In marked contrast to Pyridoxal-5-P, PLP (S) is not digested by alkaline phosphatase.

2.2 Purification Of Enzymes

4-Aminobutryate aminotransferase was purified from pig brain tissues and resolved into apoenzyme as in (4), succinic semialdehyde dehydrogenase was purified as in (5) and pyridoxal kinase as in (6). Protein concentration was determined as in (7).

A coupled assay system consisting of two purified enzymes (ie, 4-aminobutryate aminotransferase and succinic semialdehyde dehydrogense) was used to monitor the catalytic conversion of 4-aminobutryate into succinic semialdehyde (4).

2.3. Spectroscopy

Absorption spectra were recorded in a Cary model 15 spectrophotometer, and Fourier Transform 31PNMR were recorded at 72.86 MHZ on a Bruker WH-180 spectrometer. Infrared spectra were recorded in a Perkin Elmer, Model 1420 spectrophotometer.

2.4. Determination Of Dissociation Constants.

The dissociation constants of Enzyme-PLP, Enzyme-PMP and Enzyme-PLP(S) were determined by analyzing the dependence of the fraction of Enzyme (F) existing as holdenzyme as a function of the total concentration of Enzyme (Et) (8).

The method is based on enzymatic assays of samples of reconstituted anminotransferase at various concentrations of protein. (Dilution Method).

The dissociaton constant (Kd) is related to the fraction of enzyme existing at holoenzyme (F) and to the total protein concentration (Et) by means of equation.(1).

$$K_D = (1-F)^2 E_t/F \qquad (1)$$

The affinity of PLP-O-Methyl Ester, Pyridoxal-5-S and N.4. Azido-2-Nitrophenyl-Pyridoxyl-5-P for the apoenzyme was determined by enzymatic assays using the analogs of PLP as competitive inhibitors of PLP as described in (9).

2.5 Materials

PLP-Monomethylester was prepared as in (10) and Pyridoxal -5-Sulfate as in (11). All solvents and chemical reagents were reagent grade. PLP and PMP were purchased from Sigma, ATP (γ)S from Boehringer, 4-Fluoro-3-Nitrophenylazide from Molecular probes.

3. RESULTS

Table 1 summarizes the catalytic properties of the active forms of the enzyme.

Full restoration of catalytic activity is observed when the apoprotein of the enzyme 4-aminobutyrate aminotransferase is allowed to react with equimolar amounts of either pyridoxal-5-P or Pyridoxamine-5-P at neutral pH in 0.1 M triethanolamine-HCl Buffer.

Replacement of the Phosphate for a Sulphate group in the structure of the coenzyme, yield's pyridoxal-5-Sulfate, which acts as strong competitive inhibitor of the cofactor in reconstitution studies (KI = 10 nM) (Table 1).

An analog of pyridoxal-5-P which was altered in the phosphate side chain, ie, PLP monomethyl ester, binds to the apoenzyme and forms a stable complex displaying two absorption bands centered at 330 mn and 415 nm, respectively (Fig. 1). Although the spectroscopic properties of bound PLP monomethyl ester, (structure I) resembles those of the natural cofactor, no restoration of catalytic activity was detected.

Table I

Effect of PLP Analogs On The

Reconstitution Of Resolved 4-Aminobutyrate Aminotransferase (3)

Coenzyme	Activity (%)	Dissociation Constant (nM)
Pyridoxal-5-P	100	1 (1)
Pyridoxamine-5-P	100	10 (1)
Pyridoxal-5-P(S)	50	1 (1)
Pyridoxal-5-Sulfate	0	10 (2)
Pyridoxal-5-P Monomethyl Ester	0	5 (2)
N-4-Azido-2-Nitrophenyl Pyridoxyl-5-P	0	1000 (2)

(1) Determined by the Dilution Method

(2) Determined by inhibition of Reconstitution of catalytic activity.

(3) Resolved enzyme (1μM) was incubated with equimolar amounts of coenzymes at pH: 7 in 0.1M triethanolamine-HCl for 2 hours at 25° C. prior to dilution in the same buffer.

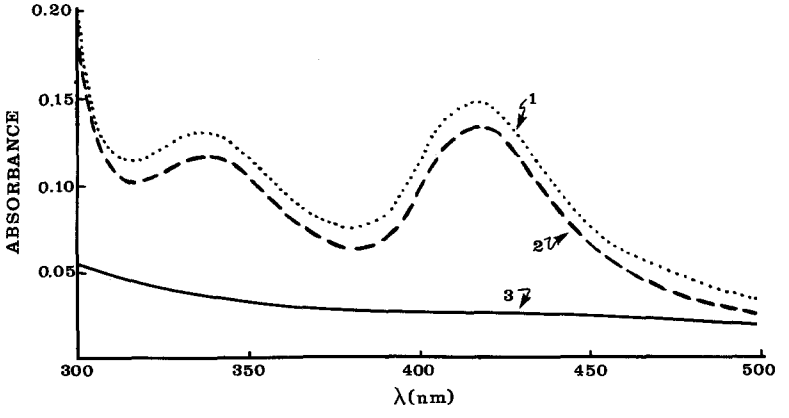
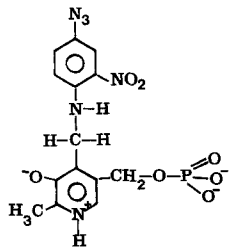
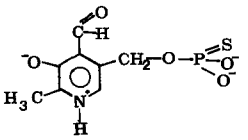


Figure 1. Absorption spectra of samples of resolved 4-aminobutyrate aminotransferase reacted with PLP-O-METHYL ESTER (—) and PLP(S) (...).

Samples of Apoenzyme (9 μM) were preincubated with either PLP-O-METHYL ESTER (9μM) or PLP(S) (9μM) in 0.1 M triethanolamine-Hl (PH7) for 2 hours at 25° C. The samples were dialized against the same buffer at 4° C. for 24 hours prior to recording the spectra.



1



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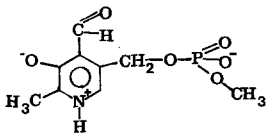

3

Judging from the absorption spectrum of bound PLP methyl ester, it appears that a Schiff's base linkage between the aldehyde group of the coenzyme and a lysyl group of the apoenzyme has been established.

However, a decrease in the negative charges of the phosphate side chain prevents the formation of a catalytically competent protein-PLP analog complex.

Apparently, the dianionic phosphate side chain is needed for correct alignment of the cofactor at the binding site. Further support for this hypothesis was derived from enzymatic studies conducted with apoenzyme reconstituted with a phosphorothioate analog of pyridoxal-5-P.

Modification of the phosphate side chain of the coenzyme by introducing a Sulfur atom convalently bound to phosphorus (Structure II) renders a phosphorothioate analog of PLP which serves as cofactor of the resolved enzyme. (Table I).

Like pyridoxal-5-P, the phosphorothioate analog reacts with the resolved enzyme to yield an absorption spectrum characterized by two intense bands centered at 330 and 415 nm, respectively (Fig. 1).

A "bulky" P-pyridoxamine derivative, N-4-Azido-2-Nitrophenyl-Pyridoxyl-5-P (NANP, Structure 3), obtained by reaction of 4-Fluoro-3-Nitrophenyl-azide with Pyridoxamine-5-P, is able to compete with the natural cofactor for the active center of the apoenzyme. In the absence of light, a reversible competion between NANP and Pyridoxal-5-P is observed (K_I = 1μM) (Table 1). However, upon illumination of a reaction mixture containing apoenzyme (10 μM) and NANP (60 μM) in 0.1 M triethanolamine-HCl, (PH7), the protein is irreversibly inactivated as shown by the results included in Figure 2.

The rate of enzyme inactivation is paralleled by a change in the absorption band of the arylazide, and the decrease in absorbance at 475 nm of the photolabeling reagent

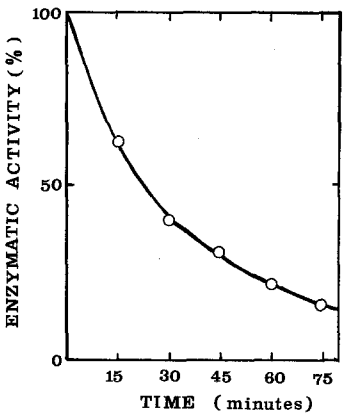

Figure 2. Change in the catalytic activity of resolved 4-aminobutyrate aminotransferase (10 μM) incubated with N-4-Azido-2-Nitrophenyl-Pyridoxyl-5-P (60 μM) and irradiated with light of 465 nm. Aliquots withdrawn from the incubation mixture were incubated with excess PLP and assayed for catalytic activity.

is due to decomposition of the arylazide derivative to generate singlet nitrene (16) as indicated in the following scheme.

$$R\text{-}N_3 \xrightarrow{h\nu} R\ddot{N}: + N_2$$

After dialysis of the modified enzyme, the presence of nitrophenyl residues covalently bound to amino acids of the protein could be detected by spectroscopic methods.

The finding that NANP recognizes the binding site of 4-Aminobutryate Aminotransferase, and the fact that upon illumination the arylazide derivative becomes irreversibly bound to the protein strongly suggests that modification of amino acid residues of the catalytic domain of the aminotransferase has taken place.

Work is underway in our laboratory to isolate the modified peptides and to compare with the A Acid sequence of the P-pyridoxyl peptide of the catalytic site.

4. DISCUSSION

Previous ^{31}P NMR measurements conducted on samples of native 4-aminobutyrate aminotransferase have shown that the chemical shift of the phosphorus signal of the coenzyme is pH independent in the range 6.05-7.5 (13). 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.

The results presented in this work indicate that the dianionic 5-Phosphate group of PLP contributes to the correct alignment of the coenzyme at the active site.

The observation that PLP (S) binds to the catalytic site of aminotransferases, together with the finding that it serves as a cofactor of w-Amino Acid Aminotransferases, opens new avenues in the study of the binding sites of vitamin B_6 dependent enzymes by means of P^{31} NMR spectroscopy. Indeed, PLP(S) exhibits a downfield shift of 21.8 ppm and PLP a chemical shift of 4 ppm at PH7 with respect to external 85% phosphoric acid.

Therefore, the possibility exists of examining the binding sites of enzymes saturated with PLP (S) and PLP, and to study the influence of effectors on those binding sites.

Another interesting aspect of the present studies is the finding that N-4-Azido-2-Nitrophenyl-Pyridoxyl-5-P acts as an efficient photolabeling reagent of 4-Aminobutyrate Aminotransferase. A characteristic feature of this arylazide derivative of Pyridoxamine -5-P, ie, its ability to recognize the cofactor binding site, can be exploited to ascertain the chemical nature of amino acid residues located in the catalytic domain.

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