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THE INTERACTION OF PYRIDOXAL PHOSPHATE WITH ASPARTATE APOAMINOTRANSFERASE

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Summary

The rate of binding of pyridoxal phosphate to the apoenzyme of pig heart cytoplasmic aspartate aminotransferase (L-aspartate: 2-oxoglutarate aminotransferase, EC 2.6.1.1) was measured by absorption spectroscopy and by formation of active enzyme. At pH 5.1 and 8.3 the binding of coenzyme follows saturation kinetics. The binding process thus involves at least two steps.

The rate of pyridoxal phosphate binding to the apoenzyme is dependent on the anion present in the pH 8.3 triethanolamine buffer. Chloride activates somewhat at very low concentrations. Phosphate and its methyl, ethyl, and phenyl esters are very effective inhibitors of the recombination in that 0.2—0.4 mM inhibit the rate of coenzyme binding by 50%. This is below the physiological concentration of phosphate. Sulfate also inhibits the rate of binding, but nitrate and acetate have little effect.

Introduction

Aspartate aminotransferase (L-aspartate: 2-oxoglutarate aminotransferase, EC 2.6.1.1) contains pyridoxal phosphate bound in Schiff base linkage to an ϵ-amino group of a lysyl residue [1]. Studies with coenzyme analogs have shown that the phosphate moiety [2—4] and probably the pyridine ring [5,6] also contribute substantially to coenzyme binding.

The kinetics of pyridoxal phosphate binding to the apoenzyme have been investigated previously [7—10], but are still poorly understood. From activation studies Banks et al. [7] concluded that pyridoxal phosphate binding goes through a two-step process. However, Arrio-Dupont [9], on the basis of activation of the enzyme and spectral changes, concluded that pyridoxal phosphate binding is a four-step process involving allosteric interaction of the subunits. Since coenzyme binding to the apoenzyme occurs very rapidly, we have used a stopped-flow apparatus to study the binding process and the intermediates involved.

Additional evidence that the phosphate moiety of pyridoxal phosphate is involved in the interaction of the coenzyme with the apoenzyme is the observation that inorganic phosphate inhibits the binding of pyridoxal phosphate [7,11,12], pyridoxamine phosphate [11,13], and other pyridoxal analogs [6] to the apoenzyme of aspartate aminotransferase. However, the affinity of the enzyme for inorganic phosphate is unknown. To gain further understanding of the role of the phosphate moiety in coenzyme binding, the rate of pyridoxal phosphate binding to the apoenzyme in the presence of varying concentrations of phosphate is reported in this study. Furthermore, the effect of other anions on the recombination process has also been studied.

Materials and Methods

Compounds. All chemicals were reagent grade. Pyridoxal 5′-phosphate, L-aspartic acid, α-ketoglutaric acid, β-NADH, Tris, triethanolamine, and phenyl phosphate were purchased from Sigma. Methyl phosphate and ethyl phosphate were obtained from K and K Laboratories, Inc. Enzyme grade $(NH_4)_2SO_4$ was from Schwarz/Mann Chemical Co., and malate dehydrogenase was from Boehringer Mannheim Corp.

Buffers were prepared by titrating Tris or triethanolamine with HCl to the appropriate pH. Measurements of pH were made at 25°C with a Radiometer Model 28 pH meter.

Enzyme. The α-subform of pig heart cytoplasmic aspartate aminotransferase was prepared according to the procedure of Martinez-Carrion et al. [14]. The apoenzyme was prepared as described previously [3]. Protein concentrations of the apoenzyme were estimated from the absorbance at 280 nm using the extinction coefficient of $63.56 \cdot 10^3$ for a subunit molecular weight of 47 000 [3]. After preparation of the apoenzyme it was used within 24 h in the stopped-flow studies and within 5 days in the inhibition studies.

Enzyme assay. The aminotransferase activity was measured by coupling the reaction with malate dehydrogenase and β-NADH according to the procedure of Karmen [15] and Amador and Wacker [16]. The assay solutions contained 6.7 mM α-ketoglutarate, 6.7 mM L-aspartate, 67 mM Tris · HCl, pH 7.4, 0.17 mg of β-NADH, and 8.3 μg of malate dehydrogenase in a total volume of 1 ml. After starting the reaction by the addition of aspartate aminotransferase, the decrease in absorbance at 340 nm was recorded every 15 s for 2 min.

Binding of pyridoxal phosphate to apoenzyme. Pyridoxal phosphate binding to the apoaminotransferase was followed spectrophotometrically and by measuring the formation of active enzyme. In measuring the rates of activation, the apoenzyme (approx. 15 μM in active sites) was incubated with pyridoxal phosphate in buffer in the dark at 20°C. At various times, 5-μl aliquots were rapidly added to 1 ml of the assay mixture.

Binding rates were measured spectrophotometrically at 20°C in a temperature-controlled cell holder with a Zeiss, PMQ II spectrophotometer. Apoenzyme (approx. 25 μM active sites) in 0.3 ml 50 mM triethanolamine, pH 8.3, was placed in a semimicro cuvette and allowed to temperature equilibrate for 10 min. Pyridoxal phosphate (15 μl) was added in an add-a-mixer (Precision Cells, Inc.), and the reaction was followed by recording the increase in absor-

bance at 350 nm at selected time intervals.

Binding rates were also measured at 20°C on a stopped-flow spectrophotometer of the Gibson-Milnes design [17]. Transmittance data were collected automatically with a 12-bit analog-digital converter (analog devices ADC-12 Q) interfaced to a digital computer (Data General Nova 1200).

First- and second-order rate constants were calculated by least squares analysis of the data.

Results

When aspartate apoaminotransferase is mixed with an equimolar amount of pyridoxal phosphate at pH 8.3, the absorbance at 388 nm due to free pyridoxal phosphate rapidly decreases, and there is a rapid increase in absorbance at 363 nm [3]. If the two are mixed at pH 5.1, there is a rapid decrease in absorbance at 388 nm and a rapid increase at 430 nm. The 363 and 430 nm absorption peaks are due to the reconstituted aspartate aminotransferase existing as a non-protonated (pH 8.3) or protonated (pH 5.1) Schiff base.

The kinetics of binding pyridoxal phosphate to aspartate apoaminotransferase was investigated with a stopped-flow apparatus. At pH 8.3 the rate of binding was measured by following the decrease in absorbance at 410 and 430 nm and the increase in absorbance at 340 and 355 nm. At these wavelengths there is adequate change in absorption upon reconstitution of the holoenzyme with a minimum amount of interference by the absorption of free pyridoxal phosphate. The binding process at pH 8.3 follows pseudo-first-order kinetics for at least three half times when there is a sufficient excess of pyridoxal phosphate over the apoenzyme (Fig. 1). Second-order kinetics is observed when there is only a small excess of coenzyme over the apoenzyme. For a given concentration of pyridoxal phosphate identical first-order rate constants are obtained for the rates measured at 340, 355, 410, and 430 nm and with 15 or 25 μM apoenzyme. A plot of the first-order rate constant (k) vs pyridoxal

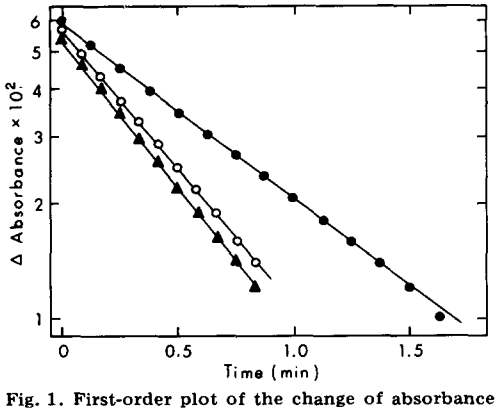

Fig. 1. First-order plot of the change of absorbance at 355 nm (○,●) or 410 nm (▲) after mixing 15 μM aspartate apoaminotransferase and 125 μM pyridoxal phosphate in either 50 mM Tris · HCl, pH 8.3 (●) or 50 mM triethanolamine · HCl, pH 8.3 (○,▲).

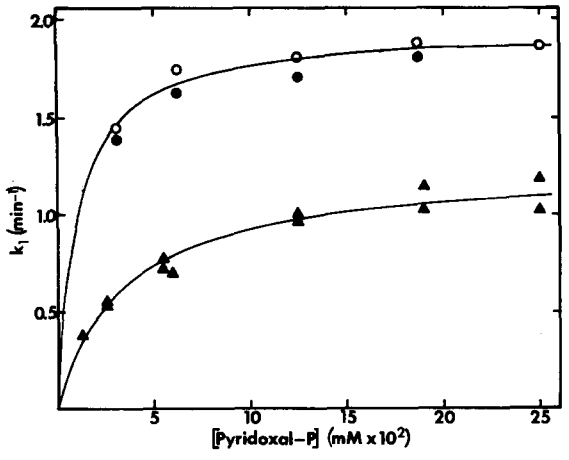

Fig. 2. The effect of pyridoxal phosphate concentration on the rate of its binding to aspartate apoaminotransferase measured by following the absorbance change at 355 nm (▲,●) or 410 nm (○) at 20° C. Pyridoxal phosphate was mixed with either 15 μM apoenzyme in 50 mM triethanolamine · HCl, pH 8.3 (○,●) or 10.5 μM apoenzyme in 50 mM Tris · HCl, pH 8.3 (▲). The solid lines represent theoretical curves based on the calculated dissociation constants and maximum velocities given in Results.

phosphate concentration shows that a maximum rate of binding is reached at high coenzyme concentrations in both 50 mM triethanolamine · HCl, pH 8.3, and 50 mM Tris · HCl, pH 8.3 (Fig. 2). When the data obtained in the Tris buffer are plotted as $1/k$ vs 1/pyridoxal phosphate, a linear relationship is obtained. These kinetic data indicate that the coenzyme binding occurs in a two-step process similar to the Michaelis-Menten mechanism for enzyme-catalyzed reactions in which the first step is rapid and the second step is rate determining. The dissociation constant for the first step (K) and the rate constant for the second step (k_2) can be calculated for such a mechanism. At pH 8.3 in Tris or triethanolamine buffer the dissociation constants are 35 μM and approx. 10 μM, respectively, and the k_2 values are 1.25 and 1.94 min^{-1}, respectively. The difference in the rate constants obtained in the two buffers is due to the reaction occurring between Tris and pyridoxal phosphate to form an imine [18]. Tris thus decreases the concentration of free pyridoxal phosphate available for reacting with the apoenzyme. All other kinetic studies at pH 8.3 were conducted in triethanolamine buffers.

Rate constants identical to those obtained in the stopped-flow experiments were obtained when the rate of formation of active aspartate aminotransferase from apoenzyme and pyridoxal phosphate was determined at pH 8.3. These rates were measured using the same concentrations of apoenzyme and coenzyme as were used in the stopped-flow studies.

The kinetics of reconstitution was also studied in 50 mM pyridine · HCl, pH 4.6 and pH 5.1. Saturation kinetics was also observed in both pyridine buffers. The rate of reconstitution at the low pH was much faster than that at pH 8.3 (k_2 was 480 min^{-1} at pH 4.6 and 600 min^{-1} at pH 5.1); however, much higher pyridoxal phosphate concentration was required to reach saturation (K equaled 0.25 mM at both pH 4.6 and 5.1).

Banks et al. [19] have shown that the monomeric aspartate apoaminotransferase binds pyridoxal phosphate more rapidly than the dimeric apoenzyme. In 10 mM triethanolamine, pH 7.3, the apoenzyme appears to dissociate into monomers at enzyme concentrations below $1 \cdot 10^{-7}$ M. We investigated the possibility that the apoenzyme dissociates more readily at pH 4.6—5.1 than at pH 8.3 thus producing monomers which interact more rapidly with pyridoxal phosphate. The apoenzyme was put over a column of Sephadex G-150 (1.5 × 84 cm) equilibrated and eluted with either 50 mM pyridine · HCL, pH 5.1, or 50 mM triethanolamine · HCl, pH 8.3. The column was calibrated with blue dextran (2 mg/ml), catalase (2 mg/ml), yeast alcohol dehydrogenase (2 mg/ml), and ovalbumin (1 mg/ml), and the elution volumes are shown in Table I. The apoaminotransferase at concentrations of 1 or 0.5 mg/ml in the two different buffers eluted in the same position as the holoenzyme. The distribution coefficients (K_d) were calculated for each protein according to the method of Siegel and Monty [20]. A plot of $K_d^{1/3}$ against the Stokes radius [20,21] of the standard proteins resulted in a straight line and a calculated Stokes radius of 37 Å for aspartate aminotransferase and its apoenzyme.

Phosphate has been shown to inhibit competitively the binding of both pyridoxal phosphate [7] and pyridoxamine phosphate [11,13] to aspartate apoaminotransferase. It was of interest to determine if other anions also intefered with reconstitution. The kinetics of binding of pyridoxal phosphate to the apoenzyme was studied at pH 8.3 in 50 mM triethanolamine buffers containing different anions. The binding was followed either spectrophotometrically at 350 nm or by measuring the activity of aliquots of the incubation mixture. In most cases we investigated the effects of the buffers on the rate of pyridoxal phosphate binding using constant concentrations of pyridoxal phosphate (97 μM) and aspartate apoaminotransferase (27 μM). The rates of absor-

TABLE I

PARAMETERS OBTAINED BY GEL FILTRATION ON SEPHADEX G-150

Gel filtration of aspartate aminotransferase and its apoenzyme on Sephadex G-150. The column was 1.5 × 84 cm, had a flow rate of 14.5 ml/h, and was run at 22°C. Each sample applied to the column was 1.5 ml, and 2-ml fractions were collected.

Material	Elution volume (ml)	Stokes radius (Å)
Blue dextran	48	
Catalase	62	52*
Yeast alcohol dehydrogenase	70	46*
Ovalbumin	91	28*
Aspartate aminotransferase	79	37
Apoenzyme		
1 mg/ml 50 mM pyridine · HCl, pH 5.1	79	
0.5 mg/ml 50 mM pyridine · HCl, pH 5.1	79	
1 mg/ml 50 mM triethanolamine · HCl, pH 8.3	78	
0.5 mg/ml 50 mM triethanolamine · HCl, pH 8.3	77	

* From Ackers [21].

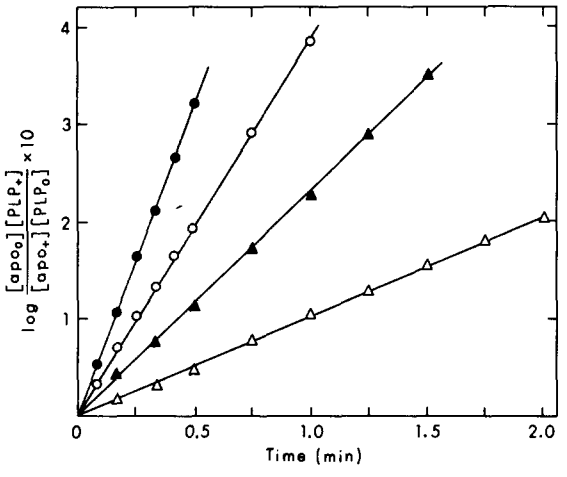

Fig. 3. Second-order plot of the change in absorbance at 350 nm versus time during the binding of 97 μM pyridoxal phosphate to 27 μM apoaminotransferase in 50 mM triethanolamine · HCl, pH 8.3, at 20°C. The triethanolamine buffer contained the following additions; ●, none; ○, 1.33 mM sulfate; ▲, 0.6 mM ethyl phosphate; and △, 0.98 mM phosphate.

bance change at 350 nm or of activation of the enzyme follow second-order kinetics as seen in Fig. 3. Apparent second-order rate constants were calculated from these data, and pseudo-first-order rate constants were obtained by multiplying the second-order rate constant by the pyridoxal phosphate concentration.

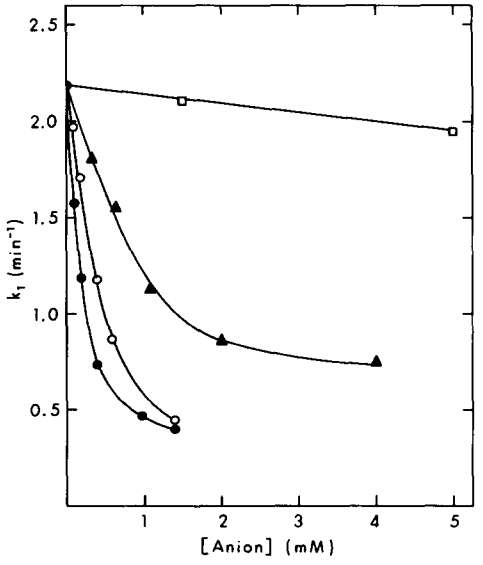

Fig. 4. The effect of various anions on the rate constant for 97 μM pyridoxal phosphate binding to 27 μM apoaminotransferase. The rates of recombination were measured by following the change in absorbance at 350 nm in 50 mM triethanolamine · HCl, pH 8.3, containing nitrate (□), sulfate (▲), ethyl phosphate (○) or phosphate (●).

TABLE II

INHIBITION OF THE RATE OF PYRIDOXAL PHOSPHATE BINDING TO ASPARTATE APOAMINOTRANSFERASE

The rate of binding 97 μM pyridoxal phosphate to 27 μM aspartate apoaminotransferase in 50 mM triethanolamine, pH 8.3, at 20°C was determined by following the increase in absorbance at 350 nm. The apparent rate constants were determined in the presence of five or six concentrations of each anion. The concentration of anion required to give 50% inhibition of the recombination rate was determined.

Anion	I_{50} (mM)
Nitrate	44
Sulfate	1.3
Phosphate	0.20
Monomethyl phosphate	0.20
Monoethyl phosphate	0.42
Phenyl phosphate	0.42

Fig. 4 shows the rate of pyridoxal phosphate binding to the apoenzyme in triethanolamine buffer containing varying amounts of nitrate, sulfate, phosphate, or ethyl phosphate. Nitrate is somewhat inhibitory; however, sulfate, phosphate, and ethyl phosphate are extremely inhibitory. Chloride activates the recombination process. There is a 2-fold increase in the rate of pyridoxal phosphate binding in going from 1 to 10 mM chloride, but the rates of recombination are the same in 10, 34, and 67 mM chloride. Table II shows the amount of various anions required to give a 50% decrease in the rate of recombination in 50 mM triethanolamine · HCl, pH 8.3. A very high concentration of nitrate is required, and this may simply be an effect of high ionic strength. Acetate is even less inhibitory in that 88 mM causes only a 35% decrease in the rate. Sulfate is a potent inhibitor. Phosphate and its methyl, ethyl, and phenyl esters are extremely good inhibitors.

Discussion

Addition of pyridoxal phosphate to the apoenzyme of aspartate aminotransferase results in the formation of reconstituted enzyme which has the same properties as the original holoenzyme [3]. The rate of the reconstitution follows saturation kinetics when determined as a function of coenzyme concentration. These data are consistent with coenzyme binding occurring in a two or more step process in which the first step is rapid and the second step is rate determining. Banks et al. [7] on the basis of their activation data also proposed a two-step process. The second slower step could be either the covalent bond formation between apoenzyme and coenzyme after initial non-covalent interaction or a conformational change which leads to active enzyme.

When coenzyme binding is followed spectrophotometrically at four different wavelengths at pH 8.3 linear pseudo-first-order plots and the same rate constants are obtained at each wavelength. The spectral changes occur at the same rate as formation of active holoenzyme. Therefore, from these data it can be concluded the rapidly formed intermediate complex and free pyridoxal phosphate have similar absorption at these wavelengths. Arrio-Dupont [9] studied the reconstitution process in bicarbonate buffer, pH 9.0, by following

the change in absorption upon the interaction of equivalent amounts of pyridoxal phosphate and apoaminotransferase. He observed a different rate when the reaction was followed at 430 nm than at 405 or 357 nm and concluded that there was a four-step binding mechanism in which a 430 nm absorbing intermediate is rapidly formed followed by the slower formation of the active 360 nm absorbing holoenzyme. We observed no differences in the rate of change of absorbance at 430 nm and at 355 nm upon the binding of a 2-fold excess of pyridoxal phosphate to the apoenzyme. It is possible that under our conditions the 430 nm form is produced too rapidly to be observed with the stopped-flow apparatus; whereas, this intermediate may be stabilized in the bicarbonate buffer.

Saturation kinetics are also observed at pH 5.1. The rate of pyridoxal phosphate binding is considerably faster at pH 5.1 than at pH 8.3. It has been shown that the coenzyme binds more rapidly to the monomeric aspartate apoaminotransferase [19]. The enhanced rate of recombination at pH 5.1 is not due to dissociation of the apoenzyme into monomers. The apoenzyme has the same Stokes radius as the holoenzyme and is therefore a dimer at both pH 8.3 and 5.1 under the conditions used in this study. The more rapid rate of recombination at the lower pH may be due to a pH-dependent protein conformation change which is not large enough to alter the Stokes radius or to an acid-catalyzed step in the reaction similar to that observed in the reaction of pyridoxal phosphate with semicarbazide [22]. Scardi [23] and Gianfreda et al. [24] also reported faster rates of reconstitution at pH 5.1 than at pH 8.3; however, the differences they obtained were much smaller and were based on determinations made at only one time after mixing coenzyme and apoenzyme.

The phosphate group of pyridoxal phosphate plays an important role in binding the coenzyme to the apoenzyme. Coenzyme binding has been shown to be inhibited by inorganic phosphate [7,11—13]. However, no reference has been made of minimum concentrations required for inhibition. Phosphate is a very effective inhibitor in that 0.2 mM results in a 50% inhibition of the rate of reconstitution of aspartate apoaminotransferase. This is well below in vivo concentrations of inorganic phosphate. Erythrocytes contain 0.8 mM [25] and brain gray matter has between 8.4 and 20.6 mM [26] inorganic phosphate. If inorganic phosphate were 0.8 mM in the cytoplasm of heart tissue, there may be as much as a 90% inhibition of the expected rate of coenzyme binding to the apoenzyme of aspartate aminotransferase in vivo.

Since methyl phosphate inhibits the rate of recombination at the same concentration as phosphate, and ethyl and phenyl phosphate are somewhat less inhibitory, there is probably no contribution of these alkyl and aryl groups toward the binding of phosphate. Phosphate and its esters all are present mainly as divalent anions at pH 8.3 and are probably binding to the apoenzyme in this ionic form. Presumably they are interacting electrostatically with the same enzymatic site that binds the phosphate moiety of the coenzyme. Several previous studies have indicated that the phosphate group of pyridoxal phosphate has an important role in binding to aspartate apoaminotransferase [2—4]. Since the phosphate group has a pK of approx. 6.2 [27], the dissociation constant would be expected to be approx. 14 times higher at pH 5.1 due to the protonation of the phosphate. In addition, the ionization of the pyridinium nitrogen

which has a pK of 8.33 [28] must be considered. If the coenzyme binds best when the pyridinium nitrogen is protonated, as proposed by Yang et al. [29], the interaction of the pyridinium portion of the coenzyme would be expected to be twice as great at pH 5.1 than at pH 8.3. Considering the contribution of both the pyridinium ring and the phosphate group, the dissociation constant for pyridoxal phosphate should be higher at pH 5.1 than at pH 8.3. The dissociation constant determined in these studies is approx. 25 times higher at pH 5.1 than at pH 8.3. The phosphate group of pyridoxal phosphate is also involved in binding this coenzyme to other apoenzymes. Phosphate and its methyl, ethyl, phenyl, and benzyl esters inhibit the binding of pyridoxal phosphate to the apoenzyme of glutamate decarboxylase [30,31]. In addition phosphate inhibits competitively the binding of pyridoxal phosphate to the apoenzymes of tyrosine aminotransferase [32] and kynurenine aminotransferase [33].

Sulfate, which is a less effective inhibitor, is presumably interacting with the same site that binds phosphate. Sulfate is extremely inhibitory in the binding of pyridoxal phosphate to glutamate apodecarboxylase [31]. The binding of pyridoxal phosphate to the apodecarboxylase was studied at low pH were phosphate is predominantly a monoanion and sulfate is a divalent anion; therefore, the observation that sulfate binds more tightly than phosphate to the apodecarboxylase is consistent with their binding as divalent anions.

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