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ACTIVATION OF APOSERINE TRANSHYDROXYMETHYLASE BY PYRIDOXAL-5'-PHOSPHATE MONOMETHYL ESTER

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SUMMARY. Aposerine transhydroxymethylase was observed to be completely reactivated by pyridoxal-P monomethyl ester. A kinetic study showed the reactivation process to be first order with respect to apoenzyme and the coenzyme analog. Reisolation of the cofactor from the holoenzyme and its identification by thin-layer and column chromatography revealed that pyridoxal-P monomethyl ester is the active coenzyme. Spectral studies indicated that enzyme reactivated with analog forms normal enzyme-substrate complexes with glycine. We conclude from these experiments that serine transhydroxymethylase can function normally with the phosphate group of the coenzyme in its monoanionic form.

A profitable way to study the function of PLP in B_6-enzymes is to use analogs of this coenzyme. This method was first applied in model systems (1, 2) and later in enzyme catalyzed reactions (3,4). These studies have shown that for PLP requiring enzymes the 4'-formyl group, the 3-hydroxy group, and the pyridine nitrogen of the coenzyme are essential for activity, whereas the 2-methyl group is not required.

One group on the coenzyme whose role has not been fully elucidated is the 5'-phosphate. There is strong evidence that this group plays an important role in binding the coenzyme at the active site. However, studies with various analogs of PLP, in which the phosphate has been replaced with other anionic groups, have failed to show any correlation between binding and enzyme activity (5,6). These observations have resulted in several investigators postulating that the phosphate group is not only involved as an anchoring point for the

Abbreviations: PLP, pyridoxal-5'-phosphate.

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coenzyme but is also involved in the catalytic mechanism (5,7).

In this paper we have tested several PLP analogs for ability to bind and form a catalytically active enzyme with aposerine transhydroxymethylase. Our results further verify the sensitivity of the formation of a holoenzyme to alterations in the phosphate side chain of PLP. Our work, however, clearly establishes that this enzyme can function at full activity with the phosphate group as a monoanion. This has not been demonstrated previously for any other PLP requiring enzyme.

EXPERIMENTAL. All solvents and chemicals were reagent grade. N,2,6-Trichloro-p-benzoquinoneimine was obtained from Aldrich. PLP monomethyl ester, prepared by the method of Pfeuffer, et al. (8), was a gift of Dr. Knut Feldmann. Pyridoxal 5'-deoxymethylenephosphonate was a gift from E. Merck, A. G. Darmstadt. Pyridoxal 5'-sulfate was prepared by the method of Kuroda (9). Serine transhydroxymethylase was purified from rabbit liver as previously described (10).

The activity of serine transhydroxymethylase was determined using allothreonine as substrate (11). The product acetaldehyde was reduced with NADH in the presence of alcohol dehydrogenase and the rate of the reaction determined by the decrease in absorbance at 340 nm. The apoenzyme was prepared by removing the enzyme-bound PLP with L-cysteine (12). All apoenzyme preparations were at least 98 percent resolved and were prepared immediately before use.

The reaction of aposerine transhydroxymethylase with PLP monomethyl ester (Structure I) was performed using two different methods. The rate of reaction of apoenzyme with I was determined from reaction solutions containing apoenzyme, and substrate. The formation of active enzyme was determined by the rate at which the product acetaldehyde was being formed in successive 15 to 30 second intervals. The percent active enzyme was determined from the difference in the rate of decrease in absorbance at 340 nm at time t and t_∞. The ln of these values were plotted against t to obtain the first order rate constant for the activation of apoenzyme. For those experiments where I was reisolated from holoenzyme the following method of activation was used. Apoenzyme, 5 to 10 mg per ml, was incubated for 2 hrs at 0° with 10 mM coenzyme analog. The excess coenzyme was removed by dialysis for 30 hrs against 6 changes of 0.01 M N-2-hydroxyethyl piperazine-N-3-propane sulfonic acid buffer, pH 7.1. These holoenzyme preparations were also used for the spectral studies of the enzyme-substrate complexes.

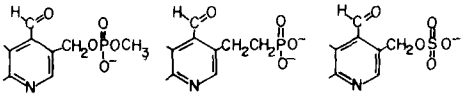
PLP monomethyl ester was isolated and identified from active holoenzyme preparations by the following method. To about 12 mg holoenzyme was added 20 µl 12% trichloroacetic acid. The precipitated protein was removed by centrifugation and 10 µl aliquots of the supernatant spotted on thin-layer cellulose sheets. Two different solvents were used to develop the chromatograms; (A) n-butanol:acetic acid:water, 60:15:15 and (B) n-butanol:acetone:water, 60:20:20. The R_f value for PLP in both solvents was 0.46. The R_f values for I were 0.67 (A) and 0.57 (B).

The location of PLP and its monomethyl ester derivative were identified by spraying the chromatograms with N,2,6-trichloro-p-benzoquinoneimine (13) and observing the blue spot in the presence of ammonia.

PLP monomethyl ester was also identified by column chromatography on DEAE-Sephadex. For this experiment 8 mg holoenzyme in 3 ml was adjusted to pH 8.4 with tris (hydroxymethyl) aminomethane. To this solution L-cysteine, 10 mM, was added and the solution placed in a boiling water bath for 1 min. After removal of the precipitate by centrifugation the supernatant was applied to a DEAE-Sephadex column, 5 mm x 50 mm, equilibrated with 0.01 M tris (hydroxymethyl) aminomethane buffer, pH 8.4. PLP and the monomethyl ester were eluted from the column by a series of buffers of increasing ionic strength and decreasing pH.

RESULTS. Aposerine transhydroxymethylase was added to an excess of three different analogs of PLP which were altered in the phosphate side chain. The enzyme formed stable complexes with two of the analogs, PLP monomethyl ester (Structure I) and pyridoxal-5'-deoxymethylenephosphonate (Structure II). These complexes could be dialyzed extensively without loss of the bound analog as evidenced by their absorption above 400 nm. Also, the addition of PLP did not lead to a rapid release of these two analogs from the enzyme.

Aposerine transhydroxymethylase did not form a stable complex with pyridoxal-5'-sulfate (Structure III). However, this compound does exhibit some affinity for the active site as evidenced by its ability to inhibit the rate of reaction of PLP with apoenzyme. A 20-fold excess of pyridoxal-5'-sulfate gives a 50 percent inhibition.



I II III

The three analogs were tested for their ability to form a catalytically active enzyme. In comparison to the activity of native serine transhydroxymethylase pyridoxal-5'-sulfate gave less than 1 percent activity and pyridoxal-5'-deoxymethylenephosphonate gave 10 percent activity. However, PLP monomethyl ester gave 100 percent activity. The remaining studies in this paper characterize the reaction of this analog with aposerine transhydroxymethylase.

Fig. 1 shows that the rate at which various concentrations of PLP monomethyl ester reactivate aposerine transhydroxymethylase is first order in

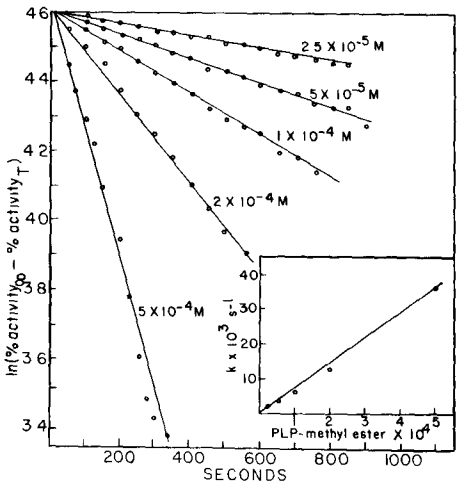


Figure 1. First order plots of the rate of formation of active enzyme from apoenzyme and PLP monomethyl ester. The reaction was started by addition of PLP monomethyl ester (concentrations given on each line) to solutions of aposerine transhydroxymethylase in an assay mixture. The inset shows the variation of the pseudo first order rate constants with increasing concentrations of PLP monomethyl ester.

enzyme concentration. The linear variation of the rate constant for reactivation as a function of the concentration of PLP monomethyl ester (Fig. 1 inset) indicates that the reactivation reaction is also first order with respect to the coenzyme analog. The rates of these reactions are at least an order of magnitude slower than the rate with PLP as coenzyme. Concentrations as low as 5×10^{-5} M PLP monomethyl ester give almost complete reactivation of aposerine transhydroxymethylase.

Identification of the enzyme-bound coenzyme analog was achieved by column and thin-layer chromatography after denaturing the PLP monomethyl ester activated holoenzyme. The upper graph in Fig. 2 shows the elution profile of samples of PLP (dashed line) and of the monomethyl ester (solid line) from a DEAE-Sephadex column by a series of buffers described in the legend to the Figure. The lower part of Fig. 2 illustrates the elution pattern of the coenzyme isolated from native serine transhydroxymethylase (dashed line) and the coenzyme from enzyme reactivated by PLP monomethyl ester (solid line).

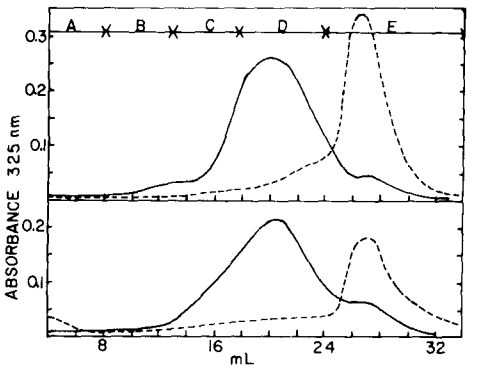


Figure 2. Elution pattern of PLP and PLP monomethyl ester from a DEAE-Sephadex column. The eluting buffers are as follows: A, 0.01 M tris (hydroxymethyl) aminomethane, pH 8.4; B, 0.05 M tris (hydroxymethyl) aminomethane, pH 8.4; C, 0.05 M tris (hydroxymethyl) aminomethane, pH 7.7; D, 0.05 M tris (hydroxymethyl) aminomethane, pH 7.4; E, 0.05 M potassium phosphate, pH 7.3. The upper curve shows the elution pattern of PLP----, and PLP monomethyl ester——. The lower pattern illustrates the elution of coenzyme isolated from native holoenzyme----, and apoenzyme reactivated with PLP monomethyl ester——.

Moreover, coenzyme analog liberated from the enzyme and authentic PLP monomethyl ester exhibited identical behavior on thin-layer chromatography in two different solvent systems. Even after incubation of analog-reactivated apoenzyme with 20 mM allothreonine for 4 hrs monomethyl ester was identified by thin-layer chromatography upon release from the enzyme.

The spectra of the enzyme and several enzyme-substrate complexes are shown in Fig. 3. Section A illustrates the enzyme containing PLP as coenzyme and section B the enzyme containing PLP monomethyl ester. The solid lines represent the spectra of both enzyme species at pH 7.1. The maximum absorption of the enzyme-bound monomethyl ester is shifted to a shorter wavelength by about 15 nm compared to the enzyme containing PLP. However, upon addition of glycine (dashed line) and tetrahydrofolate (dotted line) the spectra of the two enzyme species become essentially identical. For instance, the enzyme-glycine spectra exhibit a major peak at 425 nm and minor peaks at 343 and 495 nm. The structure and the order of appearance of the complexes in the reaction mechanism have previously been discussed (14).

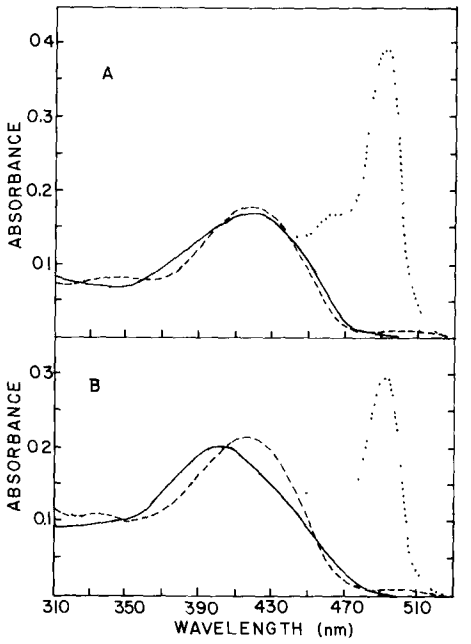


Figure 3. Spectra of serine transhydroxymethylase in the absence and presence of substrates. A: Spectrum of native serine transhydroxymethylase (1.8 mg/ml)——; spectrum of enzyme after addition of 50 mM glycine, ----; spectrum after further addition of tetrahydrofolate (0.1 mM)····. B: Spectrum of enzyme reactivated with PLP monomethyl ester (1.7 mg/ml)——; spectrum after addition of glycine (50 mM)----; spectrum after supplementary addition of tetrahydrofolate (0.1 mM)····.

The K_m for allothreonine as substrate was found to be the same for enzyme preparations containing PLP and monomethyl ester, respectively.

DISCUSSION. The data presented in this paper clearly indicate that serine transhydroxymethylase can function normally with the phosphate group of the bound PLP as a monoanion. This is shown by the full recovery of activity of the apoenzyme when reconstituted with PLP monomethyl ester. Our data also indicate that the rate at which this coenzyme analog binds at the active site is considerably slower than the rate for PLP (Fig. 1). Monomethyl ester bound as aldimine shows a spectral shift to shorter wavelengths (solid line, Fig. 3) suggesting some distortion in binding of this analog at the active site. The addition of substrates to the enzyme-bound monomethyl ester,

however, result in normal behavior, as indicated by the spectra of enzyme-substrate complexes (dashed and dotted lines in Fig. 3) and the K_m values for allothreonine.

It could be argued that the reactivation capacity of PLP monomethyl ester is due to an enzyme catalzyed hydrolysis of the ester bond at the active site to form PLP. Our data, however, suggest that this is not the case since upon removal of the coenzyme analog from the active enzyme it was identified as PLP monomethyl ester by thin-layer and column chromatography (Fig. 2). This is also true for an enzyme which had undergone several catalytic cycles with allothreonine as substrate.

The only previous examples of PLP enzymes showing appreciable activity with an analog containing a single negative charge at the 5' position are arginine decarboxylase and tryptophanase (6). Compared to their normal activity these two enzymes show 23 and 10 percent activity with pyridoxal-5'-sulfate (6). For arginine decarboxylase the pH optimum is pH 5.0 where the phosphate group of the bound PLP may normally be a monoanion. For tryptophanase the pH optimum is 8.0 where one would expect the phosphate to be a dianion. Since the pyridoxal-5'-sulfate was not reisolated from these two enzymes the possibility that the activity was due to an impurity cannot be ruled out.

Aspartate transaminase does not show activity with any PLP analog having a single negative charge in the 5'-position. For this enzyme NMR spectra have shown that the phosphate exists as a dianion from pH 5 to 9 (15).

We feel that PLP monomethyl ester will prove a better analog for investigating the activity of a monoanionic 5' side chain than pyridoxal-5'-sulfate because its structure more closely resembles the natural coenzyme.

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