INHIBITION OF ACTIVITIES OF ASPARTATE AMINO-TRANSFERASE AND TRYPTOPHANASE BY EXCESS BINDING OF PYRIDOXAL 5'-PHOSPHATE

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Pyridoxal 5'-phosphate (PLP), in high concentrations, was found to bind to lysine residues at non-catalytic sites of mammalian aspartate aminotransferase (GOT) and *E. coli* tryptophanase. This excess binding of PLP caused significant decreases in the activities of these enzymes. The concentrations of PLP required for the 50% inhibition of GOT and tryptophanase were 2.5 mM and 6.3 mM, respectively. Pyridoxal (PL) also combined with these enzymes by forming SCHIFF's bases with lysine residues at the catalytic as well as non-catalytic sites. The concentration of PL necessary for the 50% inhibition of GOT activity was 5 mM, indicating that the inhibitory action of PL is lower than that of PLP.

Pyridoxal 5'-phosphate (PLP) is known to be required in a variety of enzymatic transformations of amino acids, such as transamination, α,β -elimination and decarboxylation, etc., in which it participates as coenzyme through formation of Schiff's base with the ϵ -amino group of a specific lysine residue of apoprotein (1, 2). However, PLP also combines with lysine residue(s) of non-specific site(s) through similar Schiff's base linkage in some enzymes which do not require this coenzyme for catalysis. Hence, PLP has been used for chemical modification of these enzymes as a mean of identifying functional amino acid residue(s) that have influence on the activities. Hitherto, the action of PLP has been studied in the following enzymes: bovine liver glutamic dehydrogenase (3, 4), *Candida utilis* 6-phosphogluconic dehydrogenase (5), pyruvate kinase (6), glyceraldehyde 3-phosphate dehydrogenase (7, 8), aldolase (9), phosphofructokinase (10), and phosphoglucose isomerase (11) of rabbit muscle, hog kidney D-amino acid oxidase (12), AMP deaminase (13), acetohydroxy acid isomero-reductase (14), and rabbit liver and kidney fructose 1,6-diphosphatase (15, 16).

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PLP also binds to bovine plasma albumin (17, 18) through at least three types of binding sites, all involving ϵ -amino groups of lysine residues.

In PLP-dependent enzymes such as aspartate aminotransferase (GOT) and tryptophanase, the coenzyme is present as the Schiff's base linked to the ϵ -amino group of the specific lysine residue at the catalytic center. However, many lysine residues also exist at non-catalytic sites of these enzymes (19, 20), and PLP should bind to these non-specific lysine residues. Until now, such nonspecific binding of excess PLP and the effect to be resulted from the binding have not been studied in detail.

This paper deals with the influence of the non-specific binding of PLP on the catalytic activities of GOT and tryptophanase.

EXPERIMENTAL PROCEDURES

Materials. Crystalline bovine serum albumin, pyridoxal (PL) and pyridoxal 5'-phosphate (PLP) were purchased from the Sigma Chemical Co., U.S.A. Other chemicals were obtained from other commercial sources. Cytoplasmic aspartate aminotransferase (GOT_s) was purified from pig heart muscle according to the method of WADA and MORINO (21). ApoGOT_s was prepared from the holo-enzyme by the procedure of TURANO *et al.* (22). Apotryptophanase was obtained from *E. coli* B/it 7-A according to the procedure of NEWTON *et al.* (23).

Methods. Activities of GOT_s and tryptophanase were measured by the methods of WADA and SNELL (24) and NEWTON and SNELL (25), respectively. Protein concentrations were determined by the method of LOWRY *et al.* (26) using crystalline bovine serum albumin as a standard. Reduction of PLP-enzyme complexes with sodium borohydride was carried out as follows: after incubation of 2 ml of enzyme solution with an appropriate amount of PLP for 1 hr at 37°C in 0.1 M potassium phosphate buffer (pH 5.3), the enzyme solution was cooled to 0°C and 2 mg of finely divided sodium borohydride was then added to it. After standing for 15 min, the foam generated during reduction was removed by centrifugation. The supernatant was dialyzed in the cold against the same buffer for 24 hr. When PL was used instead of PLP, experiments were carried out in 0.1 M potassium phosphate buffer, pH 8.0. Absorption and fluorescence spectra were recorded with a Shimadzu multipurpose recording spectrophotometer 50L equipped with a double beam fluorometry attachment model 3.

RESULTS AND DISCUSSION

Effects of excess binding of PLP aspartate aminotransferase

a) *Fluorescence spectra of aspartate aminotransferase in the presence of excess pyridoxal 5'-phosphate.* In order to obtain information about the interaction between PLP and ϵ -amino groups of lysine residues at non-catalytic sites, fluores-

cence spectra of GOT in the presence of excess PLP were studied (Fig. 1). The enzyme incubated with different concentrations of PLP showed emission spectra having a peak at 515 nm when excited at 420 nm. Schiff's base derivatives

Fig. 1. Fluorescence spectra of aspartate aminotransferase excited at 420 nm in the presence of excess pyridoxal phosphate (PLP). After incubation of apoGOT (0.01 mM) with various concentrations of PLP (0.02, 0.2, 2.5 and 10 mM) in 0.1 M K-phosphate buffer (pH 5.6) at 37°C for 1 hr, fluorescence spectra excited at 420 nm of these enzyme solutions were recorded. Curve 1, apoenzyme; Curve 2, holoenzyme; Curve 3, apoenzyme + PLP (0.2 mM); Curve 4, apoenzyme + PLP (2.5 mM); Curve 5, apoenzyme + PLP (10 mM). Curves 1'-5' show the fluorescence spectra of PLP, the concentrations corresponding to Curve 1-5.

of PLP are known to absorb maximally near 420 nm (27), and also to show an emission peak near 500 nm when excited at 420 nm (28). As seen in Fig. 1, the fluorescence intensity of GOT at 515 nm increased with increasing PLP concentrations, indicating that PLP could interact with ϵ -amino groups of lysine residues located at the non-catalytic site.

b) *Absorption spectra of reduced aspartate aminotransferase-PLP complex.* Since holoenzyme-PLP complex is dissociated to holoenzyme and PLP by dilution or sufficient dialysis, fixation of PLP at the holoGOT-PLP complex was carried out by reduction of this complex with sodium borohydride. The resulting reduced holoGOT-PLP complex showed its absorption maximum at 325 nm (Fig. 2), the intensities of which increased with increasing concentrations of PLP. Those

Fig. 2. Absorption spectra of reduced aspartate aminotransferase-pyridoxal phosphate complexes. After incubation of apoGOT with various concentrations of PLP in 0.1 M K-phosphate buffer (pH 5.3) at 37°C for 1 hr, the incubated solutions were cooled to 0°C and reduced with sodium borohydride. The reduced enzyme-PLP complexes were dialyzed against the same buffer in the cold for overnight. The absorption spectrum of inner liquid was determined using the outer liquid as a blank. Curve 1, apoenzyme (0.0675 mM); Curve 2, reduced holoenzyme (0.0675 mM); Curve 3–Curve 7, reduced enzyme-PLP complexes. The concentrations of PLP were (2) 0.135 mM, (3) 0.5 mM, (4) 1.0 mM, (5) 2.5 mM, (6) 5.0 mM and (7) 10.0 mM, respectively.

absorption spectra are similar to those of the reduced Schiff's bases of PLP with various amino acids (29).

c) *Relationship between PLP concentrations and its incorporation into aspartate aminotransferase.* It is known that GOT consists of identical subunits, each containing one mole of PLP (21). The absorption intensity of reduced GOT at 325 nm corresponds to two moles of PLP incorporated into one mole of the enzyme (see, Fig. 2, Curve 2). As mentioned above, the intensity of absorbance at 325 nm was enhanced with increasing concentrations of PLP. This fact indicates that the amounts of PLP incorporated into non-catalytic sites of GOT are closely correlated with the concentrations of PLP (Fig. 3, upper). However, no linear relationship was observed between the reciprocals of the amounts of PLP incorporated and the reciprocals of PLP concentrations. On the contrary, a linear relationship existed between the reciprocals of the PLP bound to only the non-catalytic sites (moles of PLP incorporated—2) and those of the PLP

Fig. 3. Relationship between pyridoxal phosphate concentration and its incorporation into aspartate aminotransferase. Upper. The amounts of PLP incorporated into GOT were plotted against the PLP concentrations. The amounts of PLP incorporation were estimated from the data obtained in Fig. 2. Namely, the intensity of absorbance of reduced-holoenzyme (Fig. 2, Curve 2) at 325 nm corresponds to two moles PLP incorporation into one mole GOT. Lower. Double reciprocal plots of the amounts of PLP incorporated and PLP concentrations. Solid line (1): the reciprocals of the amounts of PLP incorporated into only the non-catalytic sites of GOT (moles of total PLP incorporated—2) was plotted against the reciprocals of the PLP concentrations. Broken line (2): the reciprocals of the amounts of PLP incorporated into GOT was plotted against the reciprocals of the PLP concentrations.

concentrations (Fig. 3, lower: broken line and solid line, respectively). These results reveal that only the ϵ -amino group of the lysine residue at catalytic site has a significantly higher affinity for PLP than those at non-catalytic sites. The apparent K_d value, *i.e.*, dissociation constant of holoGOT-PLP complex estimated graphically was 3.8 mM, which is about 10^4 times the K_{coenzyme} of PLP for the catalytic site of GOT.

PL is also known to bind the apoGOT to form apoenzyme-PL complex (30).

Fixation of PL in the complex was carried out by reduction with sodium borohydride. The resulting reduced apoGOT-PL complex showed its absorption maximum at 325 nm and the intensity increased with increasing concentrations of PL similarly to the case of PLP. The double reciprocal plots of the absorbance vs. PL concentration exhibited a linear relationship. Namely, the shape of the absorbance intensity at 325 nm vs. PL concentration curve is hyperbolic. These results indicate that the affinity for PL of the lysine residue at catalytic center is substantially identical with those of lysine residues at non-catalytic sites. The apparent K_d constant of PL-GOT complex was shown graphically as 9.7 mM.

d) *Inhibition of aspartate aminotransferase activity by excess PLP or PL.* As shown in Fig. 4, GOT activity was decreased in the presence of excess PLP or PL. The concentration of PLP required for 50% inactivation was estimated as

Fig. 4. Inhibition of aspartate aminotransferase activity in the presence of excess amounts of pyridoxal 5'-phosphate or pyridoxal. The enzyme (1 mg/ml) was incubated with various concentrations of PLP in 0.1 M K-phosphate buffer (pH 5.3) at 37°C for 1 hr. To the enzyme solution cooled to 4°C, was added α -ketoglutarate (10 mM). After standing for 10 min, L-aspartate (10 mM) was added and the enzymatic reaction was carried out for 2 min at 4°C. Oxalacetate formed was measured colorimetrically as 2,4-dinitrophenylhydrazone of pyruvate. Inhibition of GOT activity by PL was studied as follows: apoenzyme was preincubated with PLP (10 μ M) and α -ketoglutarate (5 mM) in 0.1 M K-phosphate buffer (pH 7.0) at 37°C for 10 min. Successively, various concentrations of PL was added to the preincubated solution and the mixture was incubated for further 30 min at 37°C. Enzymatic reaction was started after addition of L-aspartate (5 mM) and carried out for 10 min at 37°C.

2.5 mM from the figure. This value is close to the above-mentioned apparent K_d of PLP-holoGOT complex. In the case of PL, the concentration necessary for the 50% inhibition was 5 mM.

Inhibition of tryptophanase by excess binding of PLP

In a similar manner to GOT, tryptophanase activity was also decreased in the presence of excess PLP or PL. A reduced tryptophanase-PLP complex showed an absorption maximum at 330 nm, the intensity of which increased with the amounts of PLP incorporated (Fig. 5). Figure 6 depicts the relationship between the PLP concentration and PLP incorporation. Unlike the case of GOT,

Fig. 5. Absorption spectra of reduced tryptophanase-pyridoxal 5'-phosphate complexes. After incubation of apotryptophanase with different concentrations of PLP in 0.1 M K-phosphate buffer (pH 5.3) at 37°C for 1 hr, the incubated solutions were cooled to 0°C and successively reduced with sodium borohydride. The reduced tryptophanase-PLP complexes were dialyzed against the same buffer in the cold for overnight. The absorption spectrum of inner liquid was determined using the outer liquid as a blank. Curve 1, apotryptophanase (1.78 mg/ml); Curve 2–Curve 5, reduced tryptophanase-PLP complexes. The concentrations of PLP in the incubation mixture were (2) 1.0 mM, (3) 2.0 mM, (4) 3.0 mM and (5) 7.5 mM, respectively.

the reciprocals of net PLP concentrations exhibited an almost linear correlation-ship to the reciprocals of the amounts of PLP bound to tryptophanase. This fact might suggest that, contrary to GOT, the affinity for PLP of the lysine residue at the catalytic center would not be seriously different from those of the lysine residues at non-specific site in the case of tryptophanase. Our previous observation (31), that a competitive-inhibitory effect of inorganic phosphate anion against PLP or 2-nor-2-hydroxymethyl PLP was markedly weaker in tryptophanase than that in GOT, might be consistent with the supposition. Another explanation may be had by assuming that the coenzyme binding at the catalytic site of tryptophanase results in a conformational change of the enzyme protein which favors the binding of excess PLP at non-catalytic sites.

The apparent K_d of the PLP-tryptophanase complex estimated graphically was 7.1 mM. Figure 7 shows the relationship between the PLP concentrations

Fig. 6. Relationship between pyridoxal 5'-phosphate concentrations and its incorporation into apotryptophanase. Upper: the intensity of absorbance at 330 nm of reduced tryptophanase-PLP complex plotted against the PLP concentrations. Lower: double reciprocal plots of the intensities of absorbance at 330 nm and PLP concentrations.

Fig. 7. Inhibition of tryptophanase activity by excess pyridoxal 5'-phosphate. After preincubation of apotryptophanase with various concentrations of PLP in 0.1 M K-phosphate buffer (pH 8.3) at 37°C for 20 min, L-tryptophan was added to the pre-incubated mixture and then the enzymatic reaction was carried out for 10 min. Indole formed was measured colorimetrically using N,N-dimethylaminobenzaldehyde.

and the inhibitory action. The PLP concentration required for the 50% inhibition was about 6.3 mM. This value is close to the apparent K_a constant of holotryptophanase-PLP complex. As judged from the above-mentioned facts, the inhibitory action of PLP on tryptophanase would be weaker than on GOT. Similarly to the case of GOT, excess binding of PL also exerted an appreciable inhibitory effect on tryptophanase.

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