TRANSFER OF PYRIDOXAL 5'-PHOSPHATE FROM ALBUMIN-PYRIDOXAL 5'-PHOSPHATE COMPLEX TO APO-ASPARTATE AMINOTRANSFERASE

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(Received May 25, 1973)

Pyridoxal 5'-phosphate (PLP) is known to combine with bovine serum albumin to form a (1:1) complex which scarcely dissociates, even when subjected to intensive dialysis. When this complex was incubated with apo-aspartate aminotransferase (apoGOT) for an appropriate time and the preincubated mixture then submitted to the usual GOT assay, the appearance of GOT activity was obviously confirmed, indicating that PLP was transferred from the albumin-PLP complex to apoGOT. The affinity (Km) of the albumin-PLP complex for apoGOT was 1.56 μM. Simultaneous addition of α-ketoglutarate to the preincubation mixture decreased markedly the transfer of PLP. Albumin-PLP (1:1) complex showed its absorption peak at 332 nm. Although no appreciable change was observed in the absorption spectrum of the complex when incubated with apoGOT, the fluorescence spectrum of the mixture excited at 330 nm was remarkably different from that of the complex alone. On addition of DL-erythro-β-hydroxyaspartate to the incubated mixture of albumin-PLP complex with apoGOT, a new absorption peak at 492 nm, assignable to a dead-end deprotonated Schiff base between holoGOT and the substrate analog, appeared immediately. These facts strongly suggest that PLP was transferred by interaction of the albumin-PLP complex with apoGOT to yield holoGOT-substrate Schiff base on addition of substrate.

Pyridoxal 5'-phosphate (PLP) is known to inhibit the activities of various enzymes which do not require PLP as coenzymes (1-5). In these cases, the
aldehyde group and phosphate group of PLP participate in the inhibition (6): the aldehyde group forms a Schiff base with ε-amino group(s) of lysine residue(s) of these enzymes; the phosphate group competes with phosphate-containing substrates for their association with enzymes (6, 7). Our previous paper also showed that the binding of PLP to lysine residue(s) at a noncatalytic site resulted in a significant decrease in the activity of some PLP-dependent enzymes such as aspartate aminotransferase (GOT) and tryptophanase (8). PLP is also reported to bind to bovine serum albumin (9), through at least three types of binding sites (sites I, II and III), all involving the ε-amino groups of lysine residues. The binding of PLP to site I of albumin occurs with a high specificity, namely the association constant between albumin and PLP is above $10^6 \text{M}^{-1}$ (9). The resulting albumin-PLP (1:1) complex shows its absorption peak at 332 nm. The species absorbing at 332 nm is proposed by ANDERSON et al. (10) to be a substituted aldimine, i.e., aldamine.

Thus albumin can block the active aldehyde group of PLP which plays an important role in the appearance of the inhibitory action of PLP. Accordingly, the inhibitory activity of the PLP bound to albumin should be greatly weakened. Hence, if albumin traps excess PLP and the PLP moiety of the resulting PLP-albumin complex is transferred to PLP-requiring apoenzymes without releasing free PLP, the above-mentioned inhibitory action of PLP would hardly occur in vivo. Such functions of serum albumin, if they exist, seem very interesting. This paper presents evidence concerning the possibility of direct transfer of PLP from albumin-PLP complex to apoGOT.

**EXPERIMENTAL PROCEDURES**

**Materials.** Crystalline serum albumin and pyridoxal 5'-phosphate were purchased from Sigma Chemicals Co., U.S.A. DL-Erythro-β-hydroxyaspartate was obtained from Calbiochem., 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 (11). ApoGOT$_s$ was prepared from the holoenzyme by the procedure of TURANO et al. (12).

**Methods.** Activity of GOT$_s$ was measured according to the method of WADA and SNELL (13). Protein concentrations were determined by the method of LOWRY et al. (14).

Preparation of albumin-PLP (1:1) complex: bovine serum albumin (2 μmoles) were incubated with PLP (2 μmoles) in 10 ml of 0.1 M potassium phosphate buffer (pH 7.6) at room temperature. After being allowed to stand overnight, this solution was dialyzed against the same buffer in the cold for 18 hr. This dialyzed albumin-PLP complex was used for subsequent experiments. Absorption and fluorescence spectra were recorded with a Shimadzu multipurpose recording
TRANSFER OF PLP FROM ALBUMIN-PLP COMPLEX TO ApoGOT

spectrophotometer 50L equipped with a double beam fluorometry attachment model 3.

RESULTS AND DISCUSSION

Appearance of holoGOT activity by incubation of apoGOT with albumin-PLP (1:1) complex

Albumin-PLP (1:1) complex (10^{-5} M) was incubated with apoGOT (10^{-5} M) in 0.1 M K-phosphate buffer (pH 7.6) for 1 hr at 37°C. An aliquot of the incubated mixture was transferred to the usual assay solution and the appearance of GOT activity was studied. As shown in Table 1, the incubation of apoGOT with albumin-PLP complex permitted the appearance of holoGOT activity, suggesting the transfer of PLP moiety of the complex to apoGOT. GOT is known to consist of two identical subunits, each of which has one PLP-binding site (I1). Therefore, in Table 1, the subunit concentrations of apoGOT used corresponded to 2 \times 10^{-5} M. The relative GOT activity resulting from the incubation of albumin-PLP (1:1) complex with apoGOT was about one half that of the incubation mixture treated with excess PLP in order to saturate all

<table>
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<tr>
<th>Incubation system</th>
<th>Experiment No.</th>
<th>GOT activity</th>
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<tbody>
<tr>
<td></td>
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<td>µ moles OAA\textsuperscript{a} formed/10 min</td>
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<tr>
<td>Complete\textsuperscript{b}</td>
<td>1</td>
<td>0.558</td>
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<td></td>
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<td>0.621</td>
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<tr>
<td></td>
<td>2</td>
<td>1.075</td>
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<tr>
<td>Complete — complex\textsuperscript{d}</td>
<td>1</td>
<td>0.005</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.005</td>
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\textsuperscript{a} OAA: oxaloacetate

\textsuperscript{b} Albumin-PLP (1:1) complex (5 \times 10^{-5} M) and apoGOT (5 \times 10^{-5} M) were incubated in 0.1 M K-phosphate buffer, pH 7.6, at 37°C for 1.5 hr. After appropriate dilution of the incubated mixture with the same buffer, the GOT activity was assayed. The assay system contained the diluted incubated mixture (corresponding to albumin-PLP (1:1) complex, 2 \times 10^{-8} M and apoGOT, 2 \times 10^{-8} M), \alpha-ketoglutarate (10 mM) and L-aspartate (10 mM) in 0.1 M K-phosphate buffer, pH 8.0.

\textsuperscript{c} Excess PLP (10 µM) was added to the GOT assay solution, in order to saturate all of the PLP-binding sites of apoGOT used.

\textsuperscript{d} This experiment was performed in order to estimate the holoGOT to be contaminated in the apoGOT preparation used.
the PLP-binding sites of apoGOT used. This result indicates that the transfer of PLP from the albumin-PLP (1:1) complex to apoGOT proceeded almost quantitatively, one half of the PLP-binding sites being saturated.

**Time-course study on the appearance of holoGOT activity by incubation of apoGOT with albumin-PLP complex**

After apoGOT was preincubated with albumin-PLP (1:1) complex for appropriate periods at pH 8.0, L-aspartate and α-ketoglutarate were added to the incubated mixture and then the resulting holoGOT activity was assayed. As illustrated in Fig. 1, the GOT activity reached the maximum by the 20-min preincubation. When α-ketoglutarate, one of the substrates of GOT reaction, was present in the preincubation solution, the transfer of PLP was significantly lowered (Fig. 1, lower curve). This fact is consistent with our previous observation that incorporation of PLP into the catalytic center of apoGOT was also inhibited with α-ketoglutarate (unpublished results).

![Fig. 1. Time-course study of PLP-transfer from albumin-PLP (1:1) complex to apoGOT in the presence and absence of α-ketoglutarate.](image)

**Apparent Michaelis constant of albumin-PLP (1:1) complex for apoGOT**

The apparent Michaelis constant of albumin-PLP (1:1) complex for apoGOT, *i.e.*, the concentration of the complex required for the appearance of a half maximum holoGOT activity, was estimated as follows: after preincubation of apo-
GOT with various concentrations of albumin-PLP (1:1) complex for 20 min at 37°C, L-aspartate and a-ketoglutarate were added to the preincubated mixture and the enzymatic reaction was carried out for 10 min at pH 8.0. From the double reciprocal plots of GOT activities vs. albumin-PLP complex concentrations, the apparent Michaelis constant was estimated as 1.56 µM (Fig. 2).

![Double reciprocal plots of GOT activities vs. albumin-PLP complex concentrations.](image)

Fig. 2. Double reciprocal plots of GOT activities vs. albumin-PLP complex concentrations. ApoGOT was preincubated with various concentrations of albumin-PLP (1:1) complex in 0.1 M K-phosphate buffer, pH 7.6, at 37°C for 20 min. After the preincubation, L-aspartate and a-ketoglutarate (each, 10 mM) were added and the enzymatic reaction was started.

*Changes of absorption and emission spectra of albumin-PLP (1:1) complex by incubation with apoGOT*

Albumin-PLP (1:1) complex shows an absorption peak at 332 nm (Fig. 3, Curve 2). The incubation of the complex with apoGOT did not bring about any spectral change (Fig. 3, Curve 1). On the contrary, the fluorescence spectra of the complex excited at 330 nm or 405 nm changed markedly by the incubation with apoGOT (Fig. 4). This result strongly suggests that apoGOT readily interacts with albumin-PLP complex possibly to form a 1:1:1 complex.

*Effect of DL-erythro-β-hydroxyaspartate on the incubated mixture of albumin-PLP complex with apoGOT*

HoloGOT, which absorbs maximally at 362 nm at the alkaline pH region, is known to show a new absorption peak at 492 nm when erythro-β-hydroxyaspartate is added to the PLP form of the enzyme (15). This peak is ascribed to a deprotonated Schiff base formed between the substrate analog and holoGOT,
Fig. 3. Absorption spectra of albumin-PLP (1:1) complex before and after addition of apoGOT. Curve 1, absorption spectrum of albumin-PLP (1:1) complex (5.85 × 10^{-5} M) in 0.1 M K-phosphate buffer, pH 7.6, after incubation with apoGOT (5.85 × 10^{-5} M) at 37°C for 1 hr. Curve 2, absorption spectrum of albumin-PLP (1:1) complex (5.85 × 10^{-5} M) at pH 7.6.

Fig. 4. Fluorescence spectra of albumin-PLP (1:1) complex excited at 330 nm (left) or 405 nm (right) before and after incubation with apoGOT. After incubation of albumin-PLP (1:1) complex (5.85 × 10^{-5} M) with apoGOT (5.85 × 10^{-5} M) in 0.1 M K-phosphate buffer, pH 7.6, at 37°C for 1 hr, the fluorescence spectrum of this mixture excited at 330 nm (left) or 405 nm (right) was recorded. Curve 1, fluorescence spectrum of the mixture containing albumin-PLP complex and apoGOT. Curve 2, fluorescence spectrum of albumin-PLP complex only.

a dead-end ternary complex. In the case of the incubated mixture of the albumin-PLP complex with apoGOT, the absorption peak at 332 nm shifted to 492 nm immediately after addition of the substrate analog (Fig. 5). As mentioned above, the incubated mixture of the albumin-PLP complex with apoGOT did not
show any spectral change as compared with the complex alone but the fluorescence spectrum of the mixture was significantly different from that of complex. These phenomena strongly suggest that a 1:1:1 complex of albumin-PLP-apoGOT would be formed in the incubated mixture of the albumin-PLP complex with apoGOT and the addition of substrate or an appropriate substrate analog enables

![Absorbance vs Wavelength](image)

**Fig. 5.** Effect of DL-erythro-β-hydroxyaspartate on the absorption spectrum of the incubated mixture of albumin-PLP complex with apoGOT. After incubation of apoGOT (5.27 × 10^{-5} M) with albumin-PLP (1:1) complex (5.27 × 10^{-5} M) in 0.1 M K-phosphate buffer, pH 7.6, at 37°C for 1 hr, DL-erythro-β-hydroxyaspartate (3 mM) was added to the preincubated mixture. Solid line, albumin-PLP (1:1) complex (5.27 × 10^{-5} M).

the complete transfer of PLP from the albumin-PLP complex to apoGOT to form the Schiff base between substrate or substrate analog. In fact, when the albumin-PLP complex was incubated with apoGOT across a semipermeable membrane, the appearance of GOT activity, that is, transfer of PLP from the complex to apoGOT through the semipermeable membrane, was scarcely observed even on the addition of substrate.

During the course of this study, transfer of PLP from holo-cystathionase to the catalytic site of apoGOT was reported by CHURCHICH (16). The results obtained here would present positive evidence for a PLP-carrying action of serum albumin.

According to the studies of BAKER et al. (17, 18) and OKUYAMA (19) on the distribution of vitamin B₆ among human and mammalian plasma proteins, vitamin B₆ was found mainly in α- and β-globulin fractions. However, a fair amount of the vitamin was also found to be bound with albumin, especially in the cases of
a sufferer from cirrhosis of the liver and rats after being injected with pyridoxine. These results suggest that plasma proteins including albumin serve as transporters of the vitamin to metabolic sites, or as temporary storage depots.

REFERENCES

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