Specific features in the binding modes of pyridoxal phosphate N-oxide (PLP N-oxide) to mitochondrial and cytoplasmic aspartate transaminases (GOTm and GOTs) and some characteristics of the resulting artificial holoenzymes were studied. For the formation of catalytically active artificial holoenzymes, at least 10 min incubation was necessary. No significant changes were observed in the Michaelis-Menten constants of the substrates for the PLP N-oxide enzymes, except for a markedly large \( K_m \) of aspartate for GOTs, as compared with those for the PLP enzymes. In general, the \( K_m \) values of PLP N-oxide (\( K_{oe} \)) were identical with those of PLP for both GOTm and GOTs, whereas the \( V_{max} \) values of the enzymatic reactions catalyzed by the artificial holoenzymes decreased to about one-half of those mediated by the native holoenzymes. In the case of GOTs, however, a complicated pattern was observed in the curve of the reaction rate vs. PLP N-oxide concentration, indicating a sort of negative cooperativity. Namely, the enzyme was activated in the presence of higher concentrations of PLP N-oxide. In this case, binding of 1 mole of PLP N-oxide to a certain lysine residue at a non-catalytic site was ascertained. The pH optimum of the PLP N-oxide-bound GOTs was 7.0 in K-phosphate buffer. On the other hand, the reaction of PLP N-oxide enzyme was greatly inhibited in Tris-HCl buffer (pH 7.5–9.0), and the PLP-enzyme showed a higher activity in this buffer.

1 Dedicated to Professor A. E. Braunstein for his 70th birthday (1972, May).

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Among the enzymes requiring coenzymes, vitamin B₆-dependent enzymes are particularly interesting since their catalytic activities are duplicated by their coenzyme moiety, pyridoxal 5'-phosphate (PLP), or even by pyridoxal (PL) in the absence of the specific apoproteins. Sufficient evidence indicates that both the enzymatic and the model reactions operate according to the general mechanism proposed independently by Braunstein (1) and by Snell (2) on the basis of the structural features of this coenzyme molecule.

Extensive kinetic studies on the enzyme reactions (3–9) and on model reactions (2, 10–12) have demonstrated that, even though a common pattern is involved both in each PLP-dependent enzyme reaction and in its corresponding model reaction, the reaction rate is at least 10⁶ times greater in the enzyme reaction than in the model system. Moreover, the enzymatic reactions seem to proceed through a number of discrete steps (9, 13, 14). Evidently the protein moieties of the enzymes are responsible for both the specificities and the efficiencies of the holoenzymes.

In order to elucidate the roles of functional groups of vitamin B₆ for catalyses in enzymatic as well as model reactions, various analogues were synthesized, and their activities were tested. Of these analogues, PLP N-oxide, which has been newly prepared in our laboratory (15, 16), is considered to be useful since the incorporation of the oxygen atom to the pyridine nitrogen of PLP would result in a significant change in the catalytic activity of the coenzyme and in its interaction with the apoprotein.

The previous papers from our laboratory (15, 16) have shown that this analogue exhibited markedly lower catalytic activities in several enzyme systems and the model reactions. Moreover, using this analogue, Ivanov and Karpeisky (17) have reported that the incorporation of the oxygen atom had a profound influence on the interaction between the pyridine-nitrogen of PLP and a proton-donating group of aspartate aminotransferase.

This paper describes the time course of binding of PLP N-oxide to mitochondrial and cytoplasmic aspartate aminotransferases (GOTᵐ and GOTˢ) leading to the formation of artificial holoenzymes, allosteric activation of GOTˢ caused by binding of one additional molecule of PLP N-oxide, and some characteristics of the resulting artificial holoenzymes.

EXPERIMENTAL PROCEDURES

Materials. Pyridoxal phosphate N-oxide and pyridoxamine phosphate N-oxide (PMP N-oxide) were prepared in our laboratory using the method reported earlier (15, 16). Pyridoxal phosphate, pyridoxamine phosphate, and bovine serum albumin were purchased from Sigma Chemicals Co., U.S.A. Other chemicals were obtained from commercial sources.

Cytoplasmic and mitochondrial aspartate aminotransferases (GOTˢ and
GOT$_m$) were purified from pig heart muscle according to the method of WADA and MORINO(18). ApoGOT$_s$ and apoGOT$_m$ were prepared from the holoenzymes by the procedure of TURANO et al.(19).

Methods. Activities of GOT$_s$ and GOT$_m$ were measured according to WADA and SNELL(20). Protein concentrations were determined by the method of LOWRY et al.(21) using crystalline bovine serum albumin as standard. Absorption spectra were recorded with a Shimadzu multipurpose recording spectrophotometer 50 L.

RESULTS

Some characteristics of PLP N-oxide-bound aspartate aminotransferases

a) Absorption spectra of GOT$_s$ reconstituted with PLP N-oxide. The binding of PLP N-oxide to apoGOT$_s$ was measured spectrophotometrically (Fig. 1).

![Absorption spectra of GOT$_s$ reconstituted with PLP N-oxide.](image)

At pH 5.6, the artificial holoenzyme exhibited its absorption peak at 418 nm and a shoulder at 317 nm. Since the Schiff base of PL N-oxide with valine has its absorption peaks at 418 and 317 nm(15) the absorption bands of the PLP N-oxide-bound GOT$_s$ would be ascribable to the internal Schiff base formed by the protein-bound PLP N-oxide and lysine residue. At pH 8.3 (0.1 M K-phosphate buffer), the absorption peak at 418 nm was shifted to 372 nm. When this holoenzyme was dialyzed against the same buffer, the absorption spectrum showed no peak at the region over 300 nm, indicating the resolution of PLP N-oxide occurred. At pH 5.6, however, no such resolution was observed.
b) Effect of pH on the appearance of the coenzymatic activity of PLP N-oxide in GOT<sub>s</sub> system. Changes in the enzymatic activity of PLP N-oxide-bound GOT<sub>s</sub> with pH were studied using various buffer solutions. To adjust the pH, potassium phosphate buffer (from pH 5 to 8) and Tris-HCl buffer (from pH 7.5 to 9) were used.

![Graph](image)

Fig. 2. Effect of pH on the activity of PLP N-oxide-bound GOT<sub>s</sub>. ApoGOT<sub>s</sub> was incubated with 10 μM of PLP N-oxide (circle) or PLP (square) in the following buffer solutions: from pH 5 to 8, K-phosphate buffer (0.1 M) and from pH 7.5 to 9 Tris-HCl buffer (0.1 M).

As shown in Fig. 2, the enzymatic activity of PLP N-oxide-bound GOT<sub>s</sub> in phosphate buffer increased with increasing pH, attained the maximum at pH 7.0, and then decreased rapidly. The activity of PLP-bound GOT<sub>s</sub>, the native holoenzyme, increased similarly with increasing pH, and reached a higher level in the vicinity of pH 7.0. In Tris-HCl buffer (above pH 7.5), the activities of the native and artificial holoenzymes also increased with increasing pH. The activity of the latter, however, was remarkably lower than that of the former. The lower activity of the artificial holoenzyme in a higher pH region would be ascribable to the resolution of PLP N-oxide.
Time-course study of the binding of PLP N-oxide to GOT_s and GOT_m

To compare the behavior of PLP N-oxide with that of PLP in activation of apoGOT_s and apoGOT_m, the appearance of the enzyme activities was studied on a time course under various preincubation time conditions. ApoGOT_s or apo-GOT_m, PLP N-oxide, and α-ketoglutarate in phosphate buffer (pH 7.0) were pre-incubated at 37°C for various periods (0, 5, and 10 min). After the preincubation, the enzyme reaction was started by adding aspartate. As shown in Fig. 3,

![Graph showing time course of GOT reaction](image)

when the preincubation time was less than 10 min, complicated phenomena were observed in the activity of the artificial holoenzyme formed under the different preincubation periods. When the preincubation was omitted (in Fig. 3, preincubation time 0), the enzyme activity was very low until 10 min incubation. However, the enzymatic reaction proceeded normally after 10 min preincubation. In the case of PLP, such complicated phenomena did not occur. These facts strongly suggest that the conformational change of the PLP N-oxide-apoenzyme complex (Schiff base complex) to the catalytically active artificial holoenzyme is very slow, since the rate of Schiff base formation of PL N-oxide with amino acid has been demonstrated to be more rapid than that of PL(22). In the case of
GOT$_m$, analogous patterns were observed.

**Coenzymatic activity of vitamin B$_6$ N-oxides for GOT$_s$ and GOT$_m$**

It has been observed that PLP N-oxide served as coenzyme for various vitamin B$_6$-requiring apoenzymes(15, 16). Figure 4 depicts the coenzyme activity of PLP N-oxide in comparison with that of PLP in the GOT$_m$ reaction. The $K_{\text{co}}$ value of PLP N-oxide for GOT$_m$ calculated from double reciprocal plots of the reaction velocity vs. the analogue concentration was 1.19 $\mu$M. This value was

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**Fig. 4.** Comparison of coenzymatic activities of PLP N-oxide and PLP for GOT$_m$. Mixtures of apoenzyme, $\alpha$-ketoglutarate, and various concentrations of PLP N-oxide or PLP were preincubated in 0.1 M K-phosphate buffer (pH 7.0) at 37°C. After 20 min preincubation, L-aspartate was added and the activity of the resulting holoenzyme was measured by the assay of formed oxaloacetate with 2,4-dinitrophenylhydrazine after conversion to pyruvate.
essentially the same as that of PLP. On the other hand, $V_{\text{max}}$ of PLP N-oxide-bound $\text{GOT}_m$ was ca. 45% of that of the PLP enzyme. In the case of $\text{GOT}_s$, a more complicated phenomenon was observed. At low concentrations of PLP N-oxide, the reaction velocity vs. the coenzyme analogue concentration curve gave a normal Michaelis-Menten type shape. However, in high concentrations of the analogue, a sort of allosteric activation was observed (Fig. 5). The double reciprocal plots of velocity vs. PLP N-oxide concentration gave two straight lines. In low concentrations of PLP N-oxide (below 5 µM), the $K_{co}$ value obtained from the data for 20 min reaction was 1.14 µM, which was equal to that of the native coenzyme, whereas the $V_{\text{max}}$ of PLP N-oxide enzyme was about 43% of that catalyz-
ed by the PLP enzyme. In higher concentrations of PLP N-oxide (above 5 μM), the \( K_{co} \) value of PLP N-oxide was 7.9 μM, and \( V_{max} \) was almost equal to that of the PLP enzyme. This phenomenon seems to be a sort of "negative cooperativity" defined by Haber and Koshland (23). PMP N-oxide also served as coenzyme for apoGOTs. However, the activation of GOTs with PMP N-oxide was very low, and the coenzymatic activity of PMP N-oxide was about 3.5% of that of PMP. In this case, a similar complicated phenomenon was observed in the velocity vs. analogue concentration curve (Fig. 6).

Fig. 6. Comparison of coenzymatic activities of PMP N-oxide and PMP for GOTs. The experimental conditions were the same as those in Fig. 4.
Titration of apoGOTs with PLP N-oxide

It is well known that GOT, consists of two identical subunits, each of which contains 1 mole of PLP(24). As described above, PLP N-oxide also binds to apoGOTs, exhibiting a lower coenzymatic activity than PLP and a complicated velocity vs. concentration curve at a high concentration region of PLP N-oxide.

Therefore, the binding numbers of PLP N-oxide to apoGOTs were studied by a titration method as follows. ApoGOTs, $3 \times 10^{-3}$ M in 0.1 M phosphate buffer, was titrated with PLP N-oxide, and changes in the absorbance at 410 nm were followed. As shown in Fig. 7, 2 moles of PLP N-oxide were bound to 1 mole of the apoprotein, and then additional binding of 1 mole PLP N-oxide was ascertained, suggesting that this 1 mole of PLP N-oxide can readily attach to an allosteric site to render the enzyme more active.
Reduction of PLP N-oxide-bound GOTs by sodium borohydride

In order to confirm the binding of an additional 1 mole of PLP N-oxide, apoGOTs, after treatment with PLP N-oxide, was reduced with sodium borohydride. That is, apoGOTs was incubated with 2 or 3.2 times moles of PLP N-oxide at 37°C in 0.1M phosphate buffer (pH 5.3). After 2 hr incubation, solid sodium borohydride was added to the incubation mixture, and the resulting reduced enzyme was dialyzed against the same buffer overnight. The reduced enzyme showed an absorption peak at 329 nm. The ratio of the absorption intensity of GOTs incubated with 2-times moles of PLP N-oxide to that with 3.2 times moles of PLP N-oxide was 2:3 (Fig. 8). This indicates that the binding of the additional mole of PLP N-oxide is correlated with the two $K_a$ values and allosteric activation mentioned above. No further binding of PLP N-oxide was observed even though GOTs was incubated with up to 6-fold moles of the coenzyme analogue.

Allosteric Activation of GOTs by PLP N-oxide

To demonstrate the allosteric activation of GOTs caused by the binding of an additional mole of PLP-N-oxide other than the catalytic site, the enzyme activities of GOTs bound with 2 or 3 moles of PLP N-oxide were studied. ApoGOTs (final concentration, $3 \times 10^{-5}$ M) was incubated with PLP N-oxide or PLP (final
Table 1. Effect of coenzyme concentration on the activity of resulting holoGOTs.
(The concentration of apoGOTs was 10^{-5} M)

| Coenzyme   | Incubation time (min) | Concentration (×10^{-5} M) | Oxaloacetate formed (μmoles) |
|------------|-----------------------|----------------------------|-----------------------------|
| PLP        | 2                     | 2                          | 1.90 (100)                  |
|            |                       | 3.2                        | 1.89 (99.5)                 |
|            | 5                     | 2                          | 2.92 (100)                  |
|            |                       | 3.2                        | 2.88 (98.7)                 |
| PLP N-oxide| 2                     | 2                          | 1.67 (100)                  |
|            |                       | 3.2                        | 1.78 (106.5)                |
|            | 5                     | 2                          | 2.51 (100)                  |
|            |                       | 3.2                        | 2.70 (107.5)                |

Fig. 9. Allosteric activation of PLP N-oxide-bound holoGOTs by further binding of PLP N-oxide. Artificial holoGOTs was preincubated with different concentrations of PLP N-oxide in 0.1 M K-phosphate buffer (pH 7.0) at 37°C for 10 min. L-Aspartate and α-ketoglutarate were added to the preincubation mixture. The amount of oxaloacetate formed was measured colorimetrically.

As shown in Table 1, the activity of GOT, incubated with ca. 3-fold moles of PLP N-oxide was always larger than that reconstituted with 2-fold moles of PLP N-oxide. On the other hand, PLP did not exhibit such an effect under the experimental conditions. The possibility exists that PLP N-oxide bound to an
allosteric site exerts the additional catalytic activity at the site. To test this possibility, GOT, reconstituted with 2 moles of PLP N-oxide was reduced with sodium borohydride and then one additional mole of PLP N-oxide was incorporated to an allosteric site of the reduced artificial holoenzyme. The binding was confirmed spectrophotometrically, but did not result in the appearance of any catalytic activity. On the other hand, further incubation of the artificial holoenzyme (not reduced) with PLP N-oxide enhanced the activity (Fig. 9).

**Michaelis constants of aspartate and α-ketoglutarate for PLP N-oxide-bound GOT**

Table 2 shows the Michaelis constants (K_m) of substrates for PLP N-oxide-bound GOT, and GOT, in comparison with those for the PLP-enzymes. In the case of GOT, the K_m values of α-ketoglutarate were essentially the same for both the PLP N-oxide enzyme and PLP enzyme. On the other hand, the K_m of aspartate for the PLP N-oxide enzyme was somewhat smaller than that for the native holoenzyme. In the case of GOT, the K_m value of α-ketoglutarate for PLP N-oxide enzyme was slightly smaller than that for PLP enzyme, whereas aspartate gave a markedly large K_m for PLP N-oxide enzyme as compared with that for PLP enzyme.

**Fluorescence spectra of PLP N-oxide-bound GOT**

ApoGOT shows its fluorescence at 347 nm (excitation at 288 nm) which is ascribed to a tryptophan residue(s), whereas PLP N-oxide has no fluorescence at 347 nm. Incorporation of PLP N-oxide to apoGOT resulted in a significant decrease of the fluorescence at 347 nm (Fig. 10), indicating that an energy transfer from the tryptophan residue to PLP N-oxide is very strong, and the interaction between PLP N-oxide and GOT is very tight.

**DISCUSSION**

Complicated phenomena were observed in the binding process of PLP N-oxide to apoGOT, as well as the curve of the resulting enzyme activity vs. PLP N-oxide concentration as shown in Figs. 3 and 5. Possible reasons for these phenomena are as follows: (1) The binding rate of PLP N-oxide to apoGOT,
is slower than that of PLP; (2) after the formation of the Schiff base between PLP N-oxide and the apoenzyme, the time required to adopt a catalytically active holoenzyme conformation is long compared with that of PLP enzyme; (3) binding rates of PLP N-oxide to the two active sites of the enzyme are different; (4) an oxygen atom of PLP N-oxide is released during the enzyme reaction, resulting in an increase of enzyme activity; (5) PLP N-oxide binds to an allosteric site, resulting in enhancement of the enzyme activity; (6) PLP N-oxide bound to a nonspecific lysine residue at an allosteric site serves as an additional catalytic center at the allosteric site, in addition to the normal two catalytic centers of the enzyme.

Possibility (1) would be eliminated by the higher formation rate of the Schiff
base between PLP N-oxide and amino acids(22). Possibility (3) would be excluded by the data from the titration experiments (Fig. 7). In PLP N-oxide-bound phosphorylase, release of an oxygen atom from PLP N-oxide yielding PLP was reported by HELMREICH et al.(25). This fact may suggest possibility (4).

However, this possibility can be eliminated at least in the case of GOTs. After the enzyme reaction mediated by PLP N-oxide-bound GOTs was finished, the prosthetic group was released from the enzyme by hydrolysis and subjected to paper electrophoresis. From the results, the formation of PLP or PMP from PLP N-oxide was not ascertained. This fact, in addition to our previous data from a time-course study on GOTs reaction dependent on PLP N-oxide(15), would eliminate possibility (4).

Possibility (6) can also be excluded. When the artificial holoenzyme was reduced with sodium borohydride and then incubated with various concentrations of PLP N-oxide, the reduced enzyme treated thus did not exhibit any catalytic activity.

The delay in the appearance of full activity in the incubation time course of PLP N-oxide with GOTs would be caused by possibility (2). In GOTs, the pyridine-nitrogen of PLP is assumed to interact with a proton-donating group of the apoenzyme(26). Protonation of the ring nitrogen lowers the pKₐ of the hydroxyl group of the coenzyme, converting the enzyme to its active form. On the other hand, PLP N-oxide cannot accept a proton at its ring nitrogen from the donating group because of the oxygen atom. IVANOV and KARPEISKY(17) have studied the interaction of PLP N-oxide with the proton-donating group of apoGOTs by the use of circular dichroism. According to their results, the holoenzyme showed its CD band at 295 to 300 nm which is presumably due to an induced optical activity of a tyrosyl anion. On the other hand, the intensity of the 300 nm CD band was decreased, and a new positive CD band appeared with a maximum near 275 nm in PLP N-oxide-bound GOTs. The latter maximum is closer to the absorption peak of the un-ionized tyrosyl group(17). These results offer some information on the relationship between conformational changes and activities of PLP N-oxide-bound GOTs.

Furthermore, activation of GOTs at a high concentration region of PLP N-oxide was observed in the enzymatic activity vs. PLP N-oxide concentration curve (Fig. 5). An analogous phenomenon was reported by TATE and MEISTER(27) in aspartate β-decarboxylase. They considered that pyruvate served as an allosteric activator for this enzyme. When apoGOTs was titrated with PLP N-oxide, it was observed that 2 moles of the coenzyme analogue were incorporated into the catalytic sites of the apoenzyme and then an additional 1 mole was bound to an allosteric site. The binding was closely correlated with the enhancement of activity of PLP N-oxide-bound GOTs (Table 3). It appears likely that the binding of another mole of PLP N-oxide at an allosteric site of either subunit of
GOT, renders the enzyme conformation more suitable for catalysis. Under the same experimental conditions, PLP (1-2 μM) did not exhibit such an effect. In higher concentrations (150-200 μM) a slight activation of holoGOT was observed, though much higher PLP (above 200 μM) caused an inhibition of the enzyme activity(28). After GOT, was incubated with 10 mM of PLP, the mixture was reduced with sodium borohydride and then dialyzed to eliminate excess PLP. This treatment revealed that about 16 moles of PLP were incorporated into 1 mole of GOT.

Table 3. Comparison of coenzymatic parameters of PLP and PLP N-oxide for GOTs and GOTm.

| Enzyme  | Coenzyme     | Keq (μM) | Vmax \(\times\) |
|---------|--------------|----------|-----------------|
| Got      | PLP          | 1.14     | 1.0             |
|          | PLP N-oxide  | 1.14     | 0.43            |
|          | PMP          | 8.33     | 1.0             |
|          | PMP N-oxide  | 0.25     | 0.0042          |
|          |              | 14.3     | 0.075           |
| GOTm     | PLP          | 1.19     | 1.0             |
|          | PLP N-oxide  | 1.19     | 0.45            |

* Values obtained at high concentration regions of coenzyme analogues.

The observed difference between the behaviors of PLP and PLP N-oxide in the binding to a specific allosteric site of GOT, could be due to the higher rate constant and lower dissociation constant of PLP N-oxide than those of PLP in Schiff base formation with the ε-amino group of lysine. The results of our studies using flow methods appear later in this journal(22).

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