MODE OF BINDING OF PYRIDOXAL 5'-PHOSPHATE IN RAT LIVER ORNITHINE AMINOTRANSFERASE

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Summary

1. Pyridoxal 5'-phosphate and pyridoxamine 5'-phosphate were effective for the association of apo-form II of ornithine aminotransferase [EC 2.6.1.13]; whereas other B6 derivatives, such as pyridoxal, pyridoxamine, pyridoxine and pyridoxine 5'-phosphate, had no effect on form II of this apoenzyme.

2. The pyridoxal 5'-phosphate contents of the native enzyme, and reconstituted forms I and II were determined by two different methods to be 1.5 moles, 2.5 moles and 3.3 moles of pyridoxal 5'-phosphate/mole of enzyme, respectively.

Previously we reported (1) that native ornithine aminotransferase is made up of two different apoenzymes called form I and form II. These forms are composed of a single type of subunit (2), but differ in molecular weight, specific activity and absorption spectrum. The native enzyme and apo-form I are tetramers with molecular weights of 180,000, whereas apo-form II is a dimer. Apo-form II, however, associates into a tetramer holoenzyme on addition of pyridoxal 5'-phosphate. These holoenzymes reconstituted with pyridoxal 5'-phosphate showed different absorption spectra: the 280/420 nm ratio of form I was 16.8 and that of form II was 8.1. These data suggested that the reconstituted forms I and II contained different amounts of pyridoxal 5'-phosphate and that this caused their different specific activities.

In the present work the effects of various pyridoxal 5'-phosphate derivatives on the association of apo-form II were investigated and the actual amounts of pyridoxal 5'-phosphate bound to the reconstituted holo-forms I and II were determined by two different methods. The role of pyridoxal 5'-phosphate in ornithine aminotransferase was discussed.

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MATERIALS AND METHODS

Pyridoxamine 5′-phosphate (PAMP) and pyridoxal (PAL) were obtained from Sigma Chemical Co., pyridoxine 5′-phosphate (PINP) and pyridoxamine (PAM) from Nakarai Chemicals Ltd., and pyridoxine (PIN) and acrylamide from Wako Pure Chemical Industries, Ltd. Pyridoxal 5′-phosphate (PALP) was kindly supplied by Chugai Pharmaceutical Co., Ltd. The culture medium and Saccharomyces Carlsbergensis 4288 (ATCC 9080) used for microbiological determination of B₆ derivatives were purchased from Nissui Pharmaceutical Co., Ltd. All other chemicals used were commercial products of the highest grade available.

Ornithine aminotransferase was purified by the method of Sanada et al. (3). The two apoenzymes, forms I and II, were prepared as described by Sanada et al. (1) and holo- and apo-enzyme activities were assayed as reported previously (1). Protein concentration was determined spectrophotometrically by the method of Kalckar (4). PALP was determined fluorometrically by the method of Bonavita (5) and also enzymatically using apo-ornithine aminotransferase. Unesterified B₆ derivatives, such as PIN, PAL and PAM, were determined by the microbiological method using Saccharomyces Carlsbergensis 4288 (ATCC 9080) (6). Ultracentrifugal analysis was carried out using a Hitachi 282 analytical centrifuge. Polyacrylamide gel disc electrophoresis was carried out by the method of Davis (7).

RESULTS AND DISCUSSION

As reported previously (1), apo-form II associated into a tetramer in the presence of PALP, and various factors, such as temperature, pH and protein concentration did not affect this association. The effects of other coenzyme analogues besides PALP on the association were investigated. Figure 1 showed the Schlieren patterns of apo-form II after treatment with various B₆ derivatives. The $s_{20,w}$ values of form II treated with PALP or PAMP corresponded to those of the aggregated form (1); other derivatives, such as PINP, PIN, PAL and PAM, which are not catalytic coenzymes in the transaminase reaction, had no effect on the apo-form II. Figure 2 shows the patterns of various forms of ornithine aminotransferase on polyacrylamide gel disc electrophoresis. The electrophoretic mobilities of native ornithine aminotransferase and apo-form I were similar, but apo-form II migrated further toward the anode. Holo-form II, reconstituted with PALP or PAMP, had similar electrophoretic mobility to the native enzyme, but the other derivatives did not affect the electrophoretic mobility of apo-form II. These results confirm those of ultracentrifugal analysis. As discussed in the previous paper (1), several papers on B₆ enzymes have suggested (8–13) that the coenzyme PALP or its analogues have some role in maintaining or stabilizing the quaternary structure of the apo-protein. Only the catalytic coenzymes, PALP and PAMP, were effective for association with apo-form II of ornithine aminotransferase; PINP and other unesterified B₆ derivatives were
Fig. 1. Schlieren patterns of apo-form II treated with various B₆ derivatives. Apo-form II (A, 4.0 mg/ml) in 0.05 M of potassium phosphate buffer, pH 7.5 was incubated at 20°C for 30 min with 10 mM of the following analogues: PAL (B), PAM (C), PIN (D), PINP (E), PALP (F), and PAMP (G). The rotor speed was 60,000 rpm and photographs were taken 30 min after full speed was reached. The partial specific volume (v) was taken as 0.740 in calculation of the $s_{20,W}$ value.

Fig. 2. Polyacrylamide disc gel electrophoretograms of various forms of ornithine aminotransferase. Electrophoresis was carried out in 7.5% polyacrylamide gel at a constant current of 2 mA per tube for 3 hr. Samples of 20 µg of enzyme were applied to the top of the gels. (A) native ornithine aminotransferase, (B) apo-form I, and apo-form II (C). Others conditions were as for Fig. 1. PAL (D), PAM (E), PIN (F), PINP (G), PALP (H), and PAMP (I).

ineffective. These findings indicate that the coenzyme has important roles both in catalysis and in maintaining the quaternary structure of the apoenzyme.

The different specific activities of various forms of ornithine aminotransferase may be due to the different numbers of bound catalytic PALP. To test this, we measured the actual numbers of PALP bound to the different forms of enzyme. As shown in Table 1, 1.4 moles of PALP were bound to native ornithine aminotransferase, and no other unesterified B₆ derivatives were detected. In contrast,
Table 1. Vitamin B₆ group contents of various forms of enzyme.⁺

<table>
<thead>
<tr>
<th>Form</th>
<th>Moles of pyridoxal 5’-phosphate bound/180,000 g of enzyme</th>
<th>Moles of unesterified B groups bound/180,000 g of enzymeb</th>
<th>280/420 nm ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fluorometric method</td>
<td>Enzymatic method</td>
<td>Microbiological method</td>
</tr>
<tr>
<td>Native holoenzyme</td>
<td>1.40±0.06 (4)</td>
<td>1.45±0.12 (3)</td>
<td>0.03</td>
</tr>
<tr>
<td>Apo-form I</td>
<td>0.00</td>
<td>0.19±0.10 (2)</td>
<td>0.07</td>
</tr>
<tr>
<td>Reconstituted holo-form I⁺</td>
<td>2.46±0.20 (4)</td>
<td>2.56±0.33 (3)</td>
<td>16.8</td>
</tr>
<tr>
<td>Reconstituted holo-form II⁺</td>
<td>3.27±0.49 (3)</td>
<td>3.02±0.89 (3)</td>
<td>8.1</td>
</tr>
<tr>
<td>Reconstituted holoenzymed</td>
<td>2.60±0.11 (2)</td>
<td></td>
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</tr>
</tbody>
</table>

⁺ All samples were treated with 0.05 N HCl and acid soluble fractions were neutralized with NaOH and assayed.  
⁻ Pyridoxine was used as a standard.  
⁺ Apoenzymes in 0.05 M potassium phosphate buffer, pH 7.5 were incubated with 0.1 mM PALP and 5 mM α-ketoglutarate for 5 min at 37°C and then excess PALP and α-ketoglutarate were removed by passing the mixture through Sephadex G-25 equilibrated with the same buffer.  
d Holoenzyme was reconstituted at the original protein ratio (I). Values are mean ± S.D. of experiments indicated.

the holoenzymes reconstituted from apo-form I and apo-form II contained 2.5 moles and 3.3 moles of PALP, respectively. Moreover, the holoenzyme reconstituted from the original apo-enzyme, consisting of the original amounts of the two apoenzymes, contained 2.6 moles of PALP. Furthermore, all of the PALP bound to the enzymes is catalytically active, as reported previously (I). Previously we reported (2) that all these forms of ornithine aminotransferase are oligomers composed of a single type of subunit. Therefore, the above findings indicate that each subunit has one binding site for PALP. Form II is a tetramer and it was found to contain almost four PALP, whereas reconstituted form I is also a tetramer containing three PALP. Possibly some coenzyme-like substance with absorption at 330 nm is firmly bound at the one of the PALP binding sites in form I, so that there is one less PALP in the holo-form I than in the holo-form II, as reported previously (I). However, the amounts of PALP in forms I and II were not proportional to the 280/420 nm absorption ratio, the ratio of form II being just half that of form I. Thus, since only the specific activities of the two forms are proportional to their absorption ratios, one of the bound PALP in reconstituted form I is probably buried inside the enzyme protein.

On the other hand, the holoenzyme reconstituted from a mixture of the apo-form I and form II had a PALP content of 2.6 moles, which is less than the average amount 3.0 moles of PALP in a mixture of holo-form I and form II. However, native ornithine aminotransferase contained only 1.5 moles of PALP. The low value for bound PALP in the reconstituted holoenzyme was not due to only partial saturation with PALP because the value was obtained for enzyme which had been incubated with excess PALP and then passed through Sephadex G-25. These findings show that resolution causes some irreversible conformational changes in the enzyme and that the resolved apoenzyme does not change back completely to the original native enzyme. Thus, it is postulated that in native ornithine
Fig. 3. Absorption spectra of the substance obtained from apo-form I. Apo-form I (3.4 mg/ml in 0.025 M potassium phosphate buffer, pH 7.5) was treated with 0.1 N HCl. Denatured protein was removed using a collodion bag, and the absorption spectrum was recorded (A). The medium was adjusted to neutrality with 0.1 N NaOH (B), and was made alkaline by further addition of 0.1 N NaOH (C).

aminotransferase the two different forms of enzyme may have some protein interaction with each other and that this is lost in resolution. SCHLEGEL and CHRISTEN (14) reported that a subunit carrying coenzyme markedly stabilized the structure of neighbouring subunits in cytoplasmic aspartate aminotransferase and that coenzyme-induced protein interaction occurred. It is interesting that the native enzyme has only two catalytic PALP in its molecule, although the enzyme originally had four binding sites for catalytic PALP. It seems likely that in ornithine aminotransferase, formation of the quaternary structure depends on the interaction of apo-proteins, including subunit interactions, and also on interaction with coenzyme PALP at the same time. The substance bound to the apo-form I could be separated from the enzyme in 0.05 N HCl solution. Its absorption spectrum is shown in Fig. 3. No pyridoxal PALP, PAMP or other unesterified B6 derivatives were detected in this substance (Table 1), but its absorption spectrum suggests that it is some kind of the noncatalytic B6 derivative, judging from the data reported by JOHNSON and METZLER (15), and others (16, 17). The properties of this substance require further investigation.

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REFERENCES

3) SANADA, Y., SUEMORI, T., and KATUNUMA, N. (1970): Properties of ornithine amino-


