Reconstitution of Aspartate Aminotransferase From Pig Heart

by

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The enzyme aspartate aminotransferase from pig heart has been extensively investigated in several laboratories (1,2,3). It is well established that this enzyme can be resolved and reconstituted by addition of the cofactor under suitable experimental conditions (4,5,6,7). Although the rate of restoration of aminotransferase activity can be easily followed by activity measurements, the steps involved in the process of reconstitution are not well understood. It is the purpose of this note to analyze the reconstitution of the holoenzyme when the apoenzyme is allowed to react with pyridoxal-5-phosphate. Evidence is presented that at least two different steps are involved in the process of reconstitution; the first is that of binding of PLP to the catalytic site of the enzyme; the second is presumed to be related to a rearrangement of the protein structure. The enzyme aspartate aminotransferase was purified according to the method of Jenkins et al (2). The enzyme at pH: 8.5 exhibited a symmetrical absorption band at 362 mμ, indicating that the enzyme was in the PLP form. This preparation was purified further according to the method of Martinez Carrion et al (3).

Four cytoplasmic fractions were isolated after chromatography through carboxymethyl-Sephadex C-50. The fraction of greatest specific activity (Fraction IV)

was used throughout these studies. The enzyme was resolved into apoenzyme and cofactor by the method of Scardi et al (6). The release of PMP was monitored by fluorescence spectroscopy. To this end, the enzyme at a concentration of 1 mg per ml in 0.05 M phosphate buffer (pH:6.8) was converted to the PMP form by the addition of L-aspartate (20μ mole per mg of enzyme). Then the mixture was incubated at 25°C for 30 minutes and the fluorescence spectra was recorded over the range of 340-460 mμ (Exciting wavelength 330 mμ). Under these experimental conditions, the fluorescence emitted by the PMP residues attached to the enzyme was characterized by a low quantum yield. When the pH of the solution was brought to pH:4.8 by addition of KH_2PO_4 to a final concentration of 0.5, the dissociation of the enzyme into apoenzyme and PMP was accompanied by a substantial increase in the fluorescence emitted at 390 mμ (Fig. 1). The process was completed within 60 minutes and the emission spectra obtained after completion of the reaction displayed the characteristic features of free PMP in solution (8). The change in fluorescence intensity as a function of time, which is

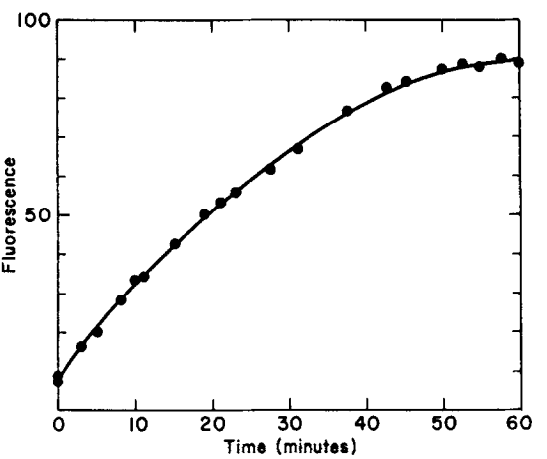

Fig. 1

Increase in fluorescence intensity at 390 mμ (excitation 330 mμ) that follows the release of Pyridoxamine-5-phosphate from the enzyme aspartate aminotransferase at pH:4.8. Experiments conducted at a protein concentration of 1 mg per ml in the fluorimeter designed in our laboratory (11).

essentially due to the release of PMP from the enzyme, can be described by the expression:

$$\ln(F_m - F_t) = k_{obs} .t + C$$

where k_{obs} is the observed first order rate constant for the removal of PMP, F_m is the fluorescence intensity value after completion of the reaction and F_t the fluorescence intensity at time t. From the experimental results of Fig. 1, k_{obs} was found to be 0.039 min^{-1}. The resulting apoenzyme was dialyzed against 0.1 M phosphate buffer (pH:7.4). After dialysis the apoenzyme showed little residual activity (1 per cent) and no fluorescence was detected at 390 mμ when excited at 330 mμ. For the reconstitution experiments, the apoenzyme at a concentration of 0.3 mg per ml was incubated with PLP at a molar ratio of 10 moles of PLP/ mole of enzyme at 30°C. Aliquots were removed at various intervals and immediately assayed for transaminase activity (9) (10). From the data included in Fig. 2, it can be seen that the process of reconstitution is relatively slow as evidenced by the fact that a recovery of 80 per cent of the original activity was obtained only after 1 hour of incubation at 30°C. In an attempt to demonstrate that the binding of PLP to the apoenzyme is a rapid process, a mixture containing 20 mg of apoenzyme was incubated with 3μ mole of PLP in 20 ml of 0.1 M phosphate buffer (pH:7.4) at 30°C. Aliquots (2 ml) were withdrawn at various times of incubation and rapidly reduced with $NaBH_4$ at 4°C for five minutes. The borohydride treated samples were dialyzed against buffer and examined both by absorption and fluorescence spectroscopy for the presence of pyridoxyl residues (8). It was found that all the pyridoxyl-apoenzyme conjugates showed the same absorbancy at 330 mμ (OD_{330}=0.2 -0.23 for 1 cm cuvettes). Furthermore, the conjugate displayed similar emission spectra when excited at 330 mμ (Table I). The fact that the pyridoxyl-apoenzyme conjugates contained approximately the same amount of pyridoxyl residues (2-3 residues per mole of enzyme) indicates that Schiff base formation occurred in the early stages of the reconstitution. Further support for this contention was obtained from experiments designed to test the ability of the conjugates

TABLE 1

Pyridoxyl-Apoenzyme Conjugates

Samples of apoenzyme were incubated with pyridoxal-5-phosphate at 30°C in 0.1 M phosphate buffer (pH:7.4). At the periods indicated aliquots were withdrawn from the incubation mixture and reduced with $NaBH_4$ at 4°C. The samples were dialyzed against 0.1 M phosphate buffer (pH:7.4) and examined for the presence of pyridoxyl residues.

Incubation Time (Minutes)	OD_{330}	F_{390} (1)	Pyridoxyl Residues / Mole of Enzyme (2)	Activity (3) (Per Cent)
3	0.21	95	2.5	2
5	0.20	95	2.4	2
10	0.21	97	2.3	1
15	0.22	96	2.5	0
30	0.21	95	2.6	0
60	0.22	100	2.6	0

(1) Fluorescence intensity measurements, (Arbitrary units)
(2) Protein concentration was determined according to Lowry et al (12) the molar extinction coefficient of the pyridoxyl residues is 8.300 $cm^2 . m\ mole^{-1}$
(3) Activity measurements performed after incubation of the conjugates with excess pyridoxal-5-phosphate.

to regain activity. If the pyridoxyl residues are bound to amino acids other than those implicated in the catalytic binding site, then it may be expected that the conjugates would regain activity upon addition of PLP. If, however, the pyridoxyl chromophores are bound to the catalytic site, then no recovery of activity would be observed upon addition of PLP. As seen in Table 1, this second result was obtained when the conjugates were incubated with ten fold

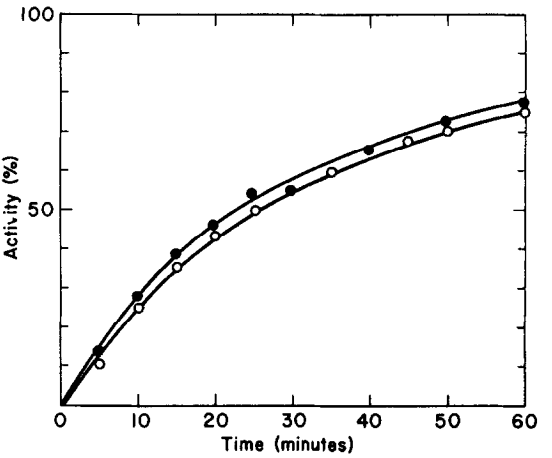

Fig. 2

Restoration of enzyme activity by preincubation at 30°C of apoenzyme with Pyridoxal-5-phosphate (PLP). Apoenzyme (0.3 mg) was incubated with 0.05 μ mole of PLP in 1 ml of 0.1 M phosphate buffer (pH:7.4). Aliquots were removed at the periods indicated in the figures and assayed for transaminase activity (10). The final concentration of enzyme in the reaction mixture was 0.2 μ g per ml. Results obtained with apoenzyme and apoenzyme treated with $NaBH_4$ in the absence of PLP are represented by closed and open circles respectively.

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excess of PLP. It should be emphasized that samples of apoenzyme reduced with $NaBH_4$ and dialyzed against buffer recover activity in the manner depicted in Fig. 2. In view of these results, it is concluded that the binding of PLP to the catalytic site of the enzyme is indeed a rapid process. It follows, therefore, that the delay in the recovery of activity may be related to a rearrangement of the protein structure. This step of the reconstitution process is currently under investigation in our laboratory.

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Abbreviations: PLP; Pyridoxal-5-Phosphate,
PMP, Pyridoxamine-5-Phosphate.

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