RECONSTITUTION OF ASPARTATE AMINOTRANSFERASE FROM ITS APOENZYME, AT pH 9.

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PLP* is known to be linked to an ε-NH_2 of a lysine of L-aspartate-2-oxoglutarate aminotransferase (E C 2,6,1,1) (1-2). The reactivation of the aspartate aminotransferase from its apoenzyme has been studied in several laboratories (3-4-5-6) and the mechanism of recovery of the enzymatic activity is not yet very well understood. With an excess of PLP, at least two steps are involved ; the first one is the binding of the coenzyme to the active site of the enzyme (5). The fixation of PLP on the lysine of the active site is a very rapid process (5).

In this paper, the reconstitution of the enzyme with equivalent amounts of PLP and apoenzyme is described. The pig heart cytoplasmic enzyme has been prepared by the method of Jenkins and Sizer (7) with the exception that we use succinate instead of maleate. The α form of the enzyme (8-9) is not prepared by the method of Martinez-Carrion, but it is obtained by an electrofocusing technique on ampholine carriers (10). The enzyme is resolved into apoenzyme and coenzyme by the method of Scardi (11), modified in the following way : the pyridoxamine form of the enzyme is formed by adding a slight excess of cysteinesulfinate, then the PMP is removed from the apoenzyme

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* No standard abbreviations : Pyridoxal-5'-phosphate : PLP.
Pyridoxamine-5'-phosphate : PMP.

by three dialysis against PO_4H_2K 1 M (half an hour for each dialysis), followed by dialysis against bicarbonate buffer pH 9, 0.05 M. At this pH the apoenzyme is stable and the bicarbonate has no effect on the enzyme reactivation.

To reconstitute the enzyme, the apoenzyme was incubated at pH 9 with two or less moles of PLP per mole of dimer apoenzyme (10^{-7} to 5.10^{-5} M) (12). The reconstitution was studied by different methods :

- recovery of enzymatic activity. The substrate solutions contained 10^{-2} M L-aspartate and 2.5 10^{-3} M α-ketoglutarate, in Tris buffer of pH 8.5 and the appearance of the enol form of oxaloacetate was measured by its absorption at 280 nm ; in the reaction mixture, the enzyme concentration was 10^{-9} to 2 10^{-9} M.

- coenzyme binding. The binding has been studied by absorption spectra of Schiff bases between 340 and 460 nm.

- quenching of fluorescence of tryptophan residues. We have observed that the quantum yield of fluorescence emission from the tryptophan residues is 0.12 in the apoenzyme and 0.055 in the enzyme at pH 9. This observation is not in agreement with that of Churchich (13), but has been carefully verified on more than ten solutions of enzyme, apoenzyme or reactivated enzyme. To study the enzyme reconstitution, we followed the variations of the intensity of the emission at 334 nm obtained by excitation at 280 nm.

The absorption spectra obtained at various times of incubation with PLP are indicated in figure 1 : it is apparent that first a Schiff base is formed, absorbing at 410-430 nm. According to the observations of Churchich (5), this base is formed with the lysine residue of the active site. In figure 2 are plotted the variations of the transaminase activity, the decrease of absorbance at 405 nm (or increase at 357 nm) and the decrease of fluorescence intensity as a function of the incubation time with PLP. One can distinguish three steps : the first one is concentration

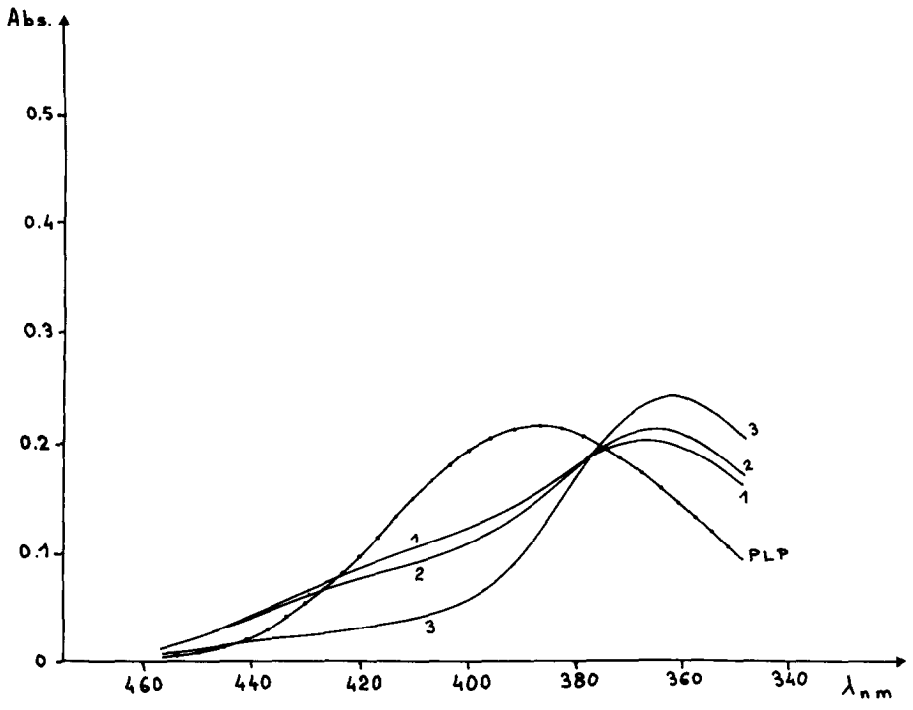

Fig. 1 - Absorption spectra of apoenzyme 1.62×10^{-5} M with PLP 3.28×10^{-5} M at various incubation times with the coenzyme :
1 : 1 minute ; 2 : 20 minutes ; 3 : 5 hours.
-•-•-•- : PLP at the same concentration and pH.

dependent (PLP or apoenzyme). If we consider the variations of the absorbance at 430 nm (fig. 3), we cannot explain the observed behaviour by a three steps mechanism. Four steps at least must be involved in the process. The first one, very rapid (ended in less than 15 seconds) is a binding of PLP on the lysine as a Schiff base which absorbs at 410-430 nm. To explain the observed variations of absorbance at 430 nm, we must suppose that first the PLP is fixed only on one of the apoenzyme protomers. The protomer with bound PLP undergoes a structural rearrangement of the protein, to give a form having enzymatic activity in which PLP absorbs at 360 nm. Then the other protomer binds the second PLP molecule and the conformation is changed

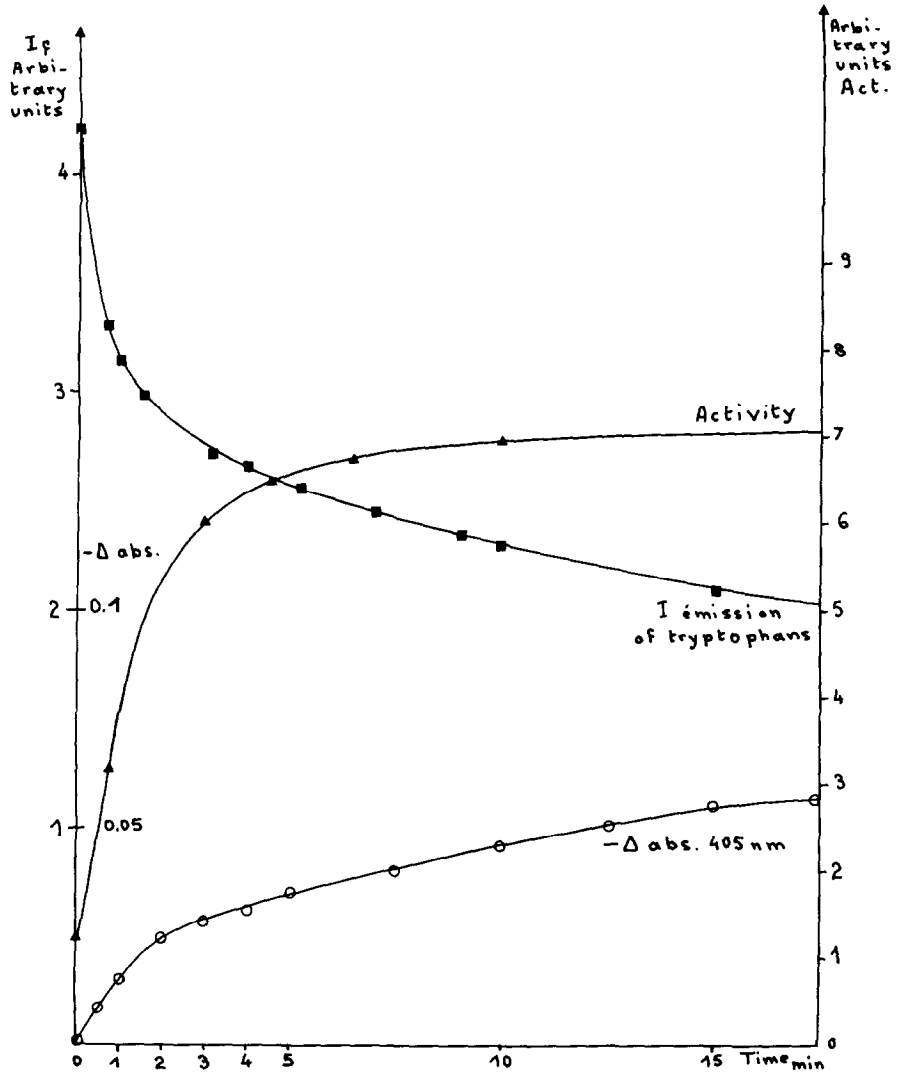

Fig. 2 - Variations of : transaminase activity ▲ , absorbance at 405 nm O , and fluorescence intensity of tryptophan residues ■ as a function of incubation time with PLP.
Apoenzyme 1.2 x 10^{-6} M ; PLP 2.26 x 10^{-6} M.

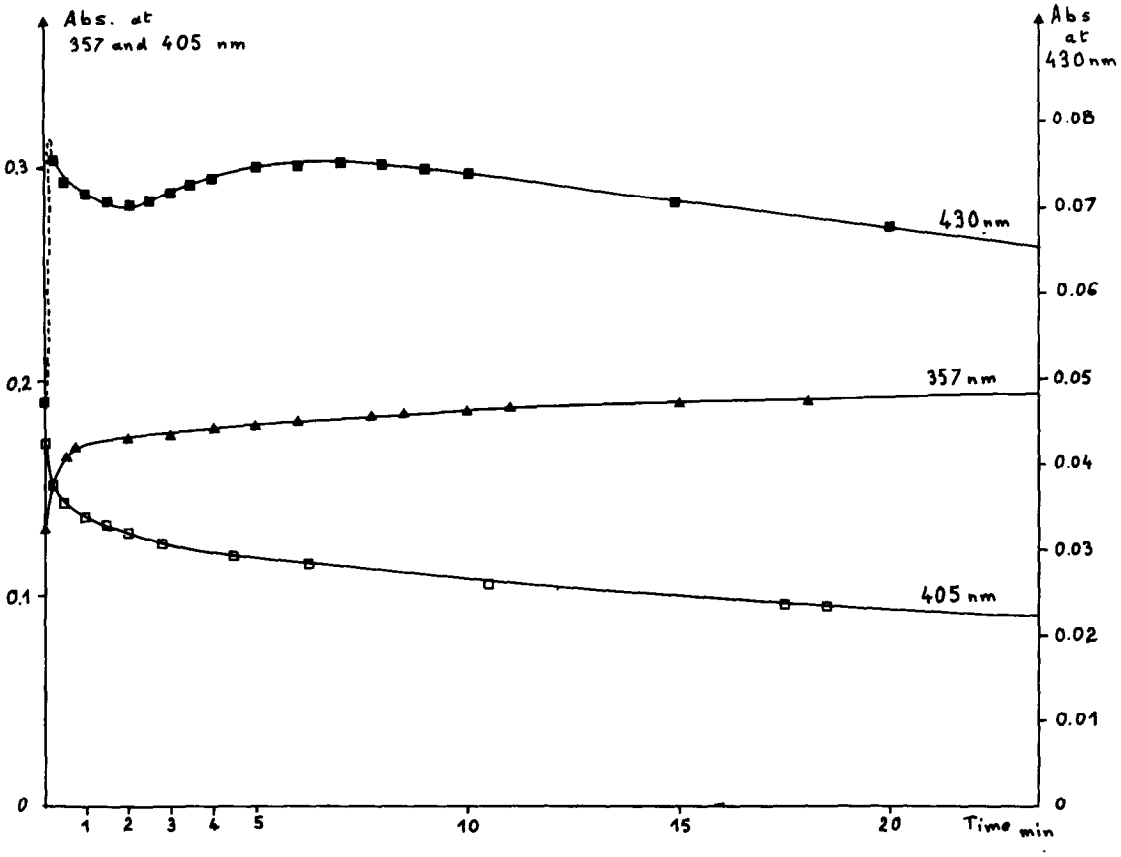

Fig. 3 - Variations of absorbance at 405, 357 and 430 nm as a function of incubation time with PLP.
Apoenzyme 1.65 x 10^{-5} M ; PLP 3.2 x 10^{-5} M.

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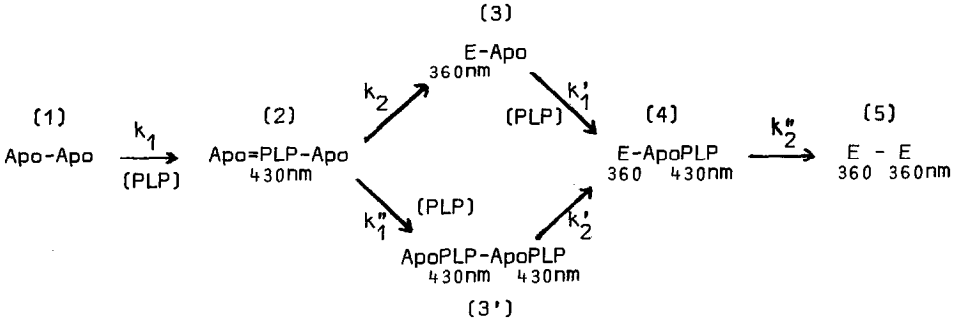
to give the fully active enzyme :



with (PLP) >> (Apo) (3-4), $k_1''(PLP) > k_2$, the steps are :

$$1 \rightarrow 2 \rightarrow 3' \rightarrow 4 \rightarrow 5$$

the authors distinguish only two steps : fixation of PLP and structural modification. With (PLP) $\leqslant$ (Apo) as in this study, $k_2 > k_1''(PLP)$, the steps are :

$$1 \rightarrow 2 \rightarrow 3 \rightarrow 4 \rightarrow 5$$

The steps of PLP fixation might be reversible.

The proposed scheme is in good agreement with the observed modifications of absorbance, fluorescence emission and activity. We are now attempting to determine the kinetic constants by a stopped-flow method for the two first steps and with an excess of apoenzyme for the others. It will be very interesting to see if the kinetic constants k_1, k_1' and k_1'' are the same or if the fixation of the second PLP molecule is modified by the first PLP bound.

REFERENCES.

1. Hughes, R.C., Jenkins, W.T. and Fisher, E.H., Proc. Nat. Acad. Sci. U.S., 48 (1962) 1615.
2. Polyanovsky, O.L. and Keil, B.A., Biokhimiya, 28 (1963) 372.
3. Banks, B.E.C., Lawrence, A.J., Vernon, C.A. and Wootton, J.F., in "Chemical and Biological aspects of pyridoxal catalysis", Perg.Press, 1963, p.197.
4. Bocharov, A.L., Ivanov, V.I., Karpeisky, M.Ya., Mamaeva, O.K. and Florentiev, V.L., Biochem. Biophys. Res. Commun., 30 (1968) 459.
5. Churchich, J.E. and Farelly, J.G., Biochem. Biophys. Res. Commun., 31 (1968) 316.
6. Severin, E.S. and Dixon, H.B.F., Biochem. J., 110 (1968) 19P.
7. Jenkins, W.T., Yphantis, D.A. and Sizer, I.W., J. Biol. Chem., 234 (1959) 51.
8. Martinez-Carrion, M., Riva, F., Turano, C. and Fasella, P., Biochem. Biophys. Res. Commun., 20 (1965) 206.
9. Martinez-Carrion, M., Turano, C., Chiancone, E., Bossa, F., Giartosio, A., Riva, F. and Fasella, P., J. Biol. Chem., 242 (1967) 2397.
10. Arrio-Dupont, M. and Duie, P., to be published.
11. Scardi, V., Scotto, P., Iaccarino, M. and Scarano, E., Biochem. J., 88 (1963) 172.
12. Banks, B.E.C., Doonan, S., Gauldie, J., Lawrence, A.J. and Vernon, C.A., European J. Biochem., 6 (1968) 507.
13. Churchich, J.E. and Harpring, L., Biochim. Biophys. Acta, 105 (1965) 575.