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Phosphorylation of threonine residue in apo-aspartate aminotransferase during the inhibition with *N*-(pyridoxyl-5'-phosphate)-L-glutamic acid

Recently we proposed[1] that a useful approach to studies of the activated states of enzymatic reactions may be based upon an investigation of substances which correspond to the intermediate coenzyme–substrate complexes. Interaction of these so-called "stage" inhibitors with apoenzyme may result in a strained conformation of the active site characteristic of a certain stage in the enzymatic reaction. A consequence of this may be the possibility of conducting reactions rather unexpected for model systems.

Experiments with *N*-(pyridoxyl-5'-phosphate)-L-amino acids revealed that these compounds serve as useful "stage" inhibitors of pyridoxal enzymes[2]. In the present paper we report some unusual transformations taking place during the interaction of *N*-(pyridoxyl-5'-phosphate)-L-glutamic acid with apo-aspartate aminotransferase, obtained from pig heart as described in ref. 3.

It appeared that in solution apo-aspartate aminotransferase (3–7 mg/ml) is rapidly inactivated at pH 5 by *N*-(pyridoxyl-5'-phosphate)-L-glutamic acid, taken in an amount of 2 moles/mole of enzyme. Only 1 mole of inhibitor was found in the supernatant after precipitation of protein from this reaction mixture. The reaction of equimolar amounts of apo-aspartate aminotransferase and *N*-(pyridoxyl-5'-phosphate)-L-glutamic acid in 5 min resulted in complete inhibition. Under identical conditions, *N*-pyridoxyl-L-glutamic acid did not inhibit apo-aspartate aminotransferase to any considerable extent. Incubation of the enzyme–inhibitor complex with excess coenzyme did not result in reactivation. Concentrated solutions of pyridoxylidene and pyridoxamino forms of aspartate aminotransferase (5–7 mg/ml) appeared to be practically insensitive to *N*-(pyridoxyl-5'-phosphate)-L-glutamic acid. Denaturation of the enzyme–inhibitor complex by heating in acidic medium (pH 3, 90°, 5 min) did not result in liberation of the inhibitor. These facts suggested that apo-aspartate aminotransferase was irreversibly inhibited by *N*-(pyridoxyl-5'-phosphate)-L-glutamic acid, the reaction occurred in the active site of the enzyme, and the phosphoester group was involved in the process of inhibition.

The denatured enzyme–inhibitor complex (40–50 mg) was subjected to pepsin digestion at pH 2, 37°, and subsequently treated with pronase at pH 8.5. After fractionation on a Dowex-50-X8 column, the hydrolysate was analyzed by paper electrophoresis (buffer: pyridine–acetic acid–water (1:5:94, by vol.), pH 4, 100 V/cm, detection with dichloroquinone–chloroimine[4], with ammonium molybdate[5], or by fluorescence under ultraviolet light). The following substances were found: (I) *N*-pyridoxyl-L-glutamic acid (an aliquot of the mixture before column fractionation); (II) phospho-L-threonine; (III) a small amount of inorganic phosphate; (IV) phosphate-containing peptide. No *N*-(pyridoxyl-5'-phosphate)-L-glutamic acid was detected. To identify phospho-L-threonine, the corresponding band of the electrophoretogram was eluted with water, the eluate hydrolyzed with 1 M HCl at 100° for 48 h, and inorganic phosphate[6] and threonine[7] were determined. The amount of phospho-L-threonine corresponds to approx. 40% of the theoretical value; about the same amount is contained in the peptide.

Hence, the inhibition of apo-aspartate aminotransferase by *N*-(pyridoxyl-5′-phosphate)-L-glutamic acid leads to phosphorylation of one of the 24 threonine residues of the enzyme. It will be noted that *N*-(pyridoxyl-5′-phosphate)-L-glutamic acid, like other phosphoric acid monoesters, is by no means a phosphorylating agent, and its phosphate bond is stable under rather drastic conditions (complete liberation of inorganic phosphate requires heating for 12 h at 110° in 6 M HCl). For this reason, the ease of the transphosphorylation in the reaction of apo-aspartate aminotransferase with *N*-(pyridoxyl-5′-phosphate)-L-glutamic acid may be the consequence of the forced proximity of the functionating active site threonine hydroxyl group with the inhibitor 5′-phosphate group, and there is some evidence in favor of the participation of one more nucleophilic groups of protein in the process.

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