AN AFFINITY LABELING REAGENT FOR PYRIDOXAL PHOSPHATE DEPENDENT ENZYMES.

F. Riva, A. Giartosio, C. Borri Voltattorni, A. Orlacchio and C. Turano.

Institutes of Biological Chemistry, Universities of Rome and Perugia and Center of Molecular Biology of C.N.R., Rome.

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SUMMARY. An analogue of pyridoxal-5'- phosphate, 4'-N-(2,4 dinitro-5-fluorophenyl) pyridoxamine-5'-phosphate, has been synthesised and has been shown to behave as an affinity labeling reagent for the apoenzymes of aspartate and tyrosine aminotransferases,tyrosine decarboxylase and tryptophanase. Of the enzymes tested only apocystathionase is not irreversibly inhibited by the reagent.

In the course of investigations on derivatives of Pyridoxal-P[1] it appeared that the position 4' can be extensively modified and substituted even with bulky groups without a marked impairment of the binding to the B_6-dependent apoenzymes (1,2). We have studied the effect of introducing a reactive function on substituents in the 4' position of the coenzyme, so as to obtain a coenzyme derivative capable of irreversible binding at the enzyme active site. We describe here one of these compounds which is indeed capable of inhibiting irreversibly a variety of Pyridoxal-P-dependent apoenzymes, and is therefore a promising reagent for structural and comparative studies of this class of enzymes.

EXPERIMENTAL

4'-N-(2,4-dinitro-5-fluorophenyl) pyridoxamine-5'-phosphate (compound I) was synthesised by reacting equimolar amounts of pyridoxamine-5'-phosphate and 1,5-difluoro-2,4-dinitrobenzene at

[1] Abbreviation: Pyridoxal-P, pyridoxal-5'-phosphate.

pH 10 in aqueous solution for two hours at room temperature. The attachment of the fluorodinitrophenyl group to the amino group, and not to the phenolic hydroxyl of pyridoxamine phosphate was indicated by the absence of the ninhydrin reaction of compound I, by its positive 2,6-dichloroquinonechloroimide test and by its spectral characteristics (Fig. 1). The 340 nm peak and the 400 nm shoulder indicate that a nitrogen and not an oxygen is bound to the fluorodinitrophenyl group (3). Furthermore the spectral variations which are observed at low and high pH values indicate that the phenolic hydroxyl and the pyridine nitrogen of the pyridoxamine moiety have not been modified (4). The non-fluorinated analogue, 4'-N-(2,4-dinitrophenyl) pyridoxamine-5'-phosphate (II) was synthesised as compound I by reacting pyridoxamine-5'-phosphate with 2,4-dinitrofluorobenzene.

The non-phosphorylated analogue of I,4'-N-(2,4-dinitro-5-fluorophenyl) pyridoxamine (III) was obtained by the action of alkaline phosphatase on I.

Details of the synthesis and purification of these compounds will be published elsewhere.

Aspartate aminotransferase (EC 2.6.1.1), the "soluble" isozyme prepared from pig heart (5), was resolved from the coenzyme according to Scardi et al. (6) and assayed according to Karmen (7). Tyrosine aminotransferase (EC 2.6.1.5) was prepared and resolved according to Hayashi et al. (8) and assayed by the method of Diamondstone (9). Apotyrosine decarboxylase (EC 4.1.1.25) was obtained from Streptococcus faecalis cells grown in the absence pyridoxine (Sigma Chem. Co.). The enzyme was extracted according to Maruyama and Coursin (10) and the activity assayed by a titrimetric method (11). Triptophanase (EC 4.1.99.1) from E. Coli (Sigma Chem. Co.) was prepared and resolved according to Okuda et al. (12) and assayed according to Morino and Snell (13), cystathionase (EC 4.2.1.15) was prepared from rat liver, assayed and resolved according to Matsuo and Greenberg (14-15).

RESULTS AND DISCUSSION

After a short incubation of I with apo aspartate transaminase, only less than 10% of the original activity is recovered upon addition of an excess of coenzyme to the apoenzyme (Table I). Instead the apoenzyme treated with II can be fully reactivated; a reactivation of more than 90% is also observed with compound III; only a slight inhibition is observed when I is incubated with the holoenzyme. The observed irreversible inhibition of apo aspartate aminotransferase therefore requires a free coenzyme binding site, the presence on the inhibitor of the phosphate group, which is known to have its own binding site at the active

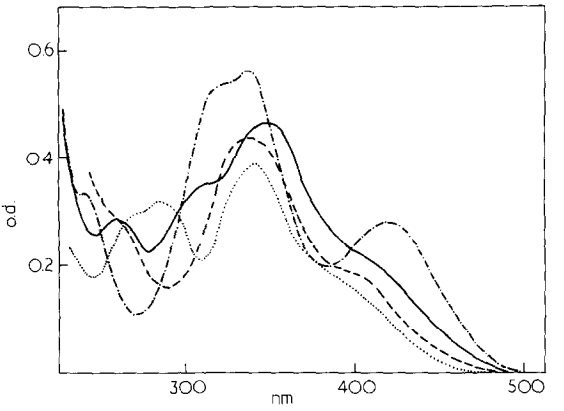

Fig. 1 - Absorption spectra of compound I (2.8×10^{-5} M)
in 0.1 M NaOH - - - -
in 0.1 M HCl .....
in 0.1 M phosphate buffer pH 7 ———
in 1 M NaOH after 1 hr. —·—·—·—·

site of aminotransferases (16), and a reactive arylating function. A specific inhibitor-apoenzyme interaction is also suggested by the low concentration of I required for inhibition. The formation of a covalent bond between inhibitor and protein is demonstrated by the following experiment: the apoenzyme was reacted for 15 minutes at pH 8 with a stoichiometric amount (based on active site concentration) of I; the mixture was then treated with 5% trichloroacetic acid, and the turbid solution was centrifuged. The precipitate, containing the denatured protein, was yellow in appearance, while no significant absorption at 340 nm due to free reagent could be detected in the supernatant. When the same experiment was performed with compound II, the precipitate was colourless and the supernatant showed the expected spectrum of II.

It appears therefore that I behaves as a typical affinity labeling reagent for apo-aspartate aminotransferase. Unfortunately a detailed analysis of the reversible and irreversible phases of binding is difficult since this reagent, as well as compound II, slowly regenerate active coenzyme when bound to the enzy-

TABLE I

Effect of compounds I, II and III on aspartate aminotransferase.

Inhibitor	Inhibitor concentration(M)	Reaction time (minutes)	Inhibition (% of untreated apoenzyme)
I	$1x10^{-5}$	5	93
I	$1x10^{-6}$	25	91
I	$1x10^{-6}$	50	94
I	$1x10^{-5}$	1	49 (°)
I	$1x10^{-5}$	40	85 (°)
I	$1x10^{-5}$	50	7 (°°)
II	$1x10^{-5}$	100	0
III	$1x10^{-5}$	1	2
III	$1x10^{-5}$	420	7
III	$2x10^{-5}$	100	7

The apoenzyme and the indicated compound were incubated at 25° C in the dark, in 0.05 M Tris-HCl buffer, pH 8. An aliquot of the reaction mixture was then transferred to a solution containing $2.5x10^{-4}$ M Pyridoxal-P and all the components for the activity assay (7) except ketoglutarate; this was added after 10 minutes, and the decrease of the optical density at 340 nm was recorded.
(°) In these experiments apoenzyme and I were incubated in 0.05 M Hepes buffer, pH 7.
(°°) In this experiment compound I was incubated with the holoenzyme.

me, in some as yet unexplained way. This is clearly shown for compound II in Fig. 2. II is able to bind reversibly to the active site of the apoenzyme, as indicated by its inhibitory action on the reactivation by pyridoxamine-5'-phosphate, but is also able by itself to reactivate slowly the enzyme. The fact that this reaction takes place with an inhibitor concentration stoichiometric with the enzyme rules out the possibility that contaminating traces of active coenzyme are responsible for the reactivation. This unexpected reaction may also explain the lack of 100%

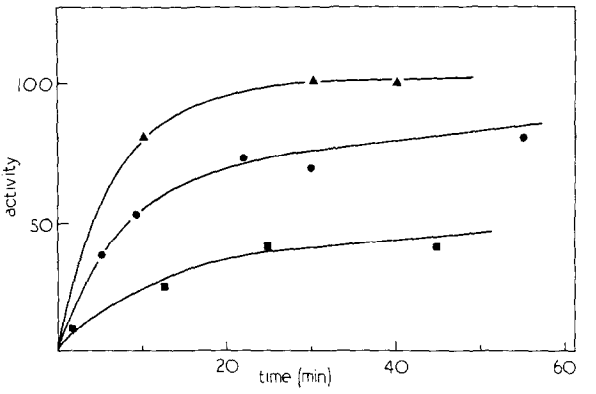

Fig. 2 - Reactivation of apo aspartate aminotransferase (active sites concentration: 10^{-6}M) in 0.05 M Tris-HCl buffer, pH 8, in the presence of 10^{-6} M pyridoxamine-5'-phosphate ▲, of 10^{-6} M pyridoxamine-5'-phosphate plus 10^{-6} M compound II ●, and of 10^{-6} M compound II alone. ■ Activities are expressed as percentage of the maximum activity.

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inactivation of the apoenzyme by I.

Compound I acts as an irreversible inhibitor also on other Pyridoxal-P dependent apoenzymes, which have a different substrate or reaction specificity, as shown in Table II. Only apo-cystathionase, among the five enzymes tested, appears to be insensitive to I, although it is capable of binding it reversibly, as shown by a competitive inhibition of the coenzyme recombination.

A common feature of the Pyridoxal-P enzymes is the lysine amino group which binds the aldehydic group of the coenzyme (16). This amino group might be the nucleophile involved in the irreversible binding of I. However we think that this is a remote possibility and that another group at the active site is involved for the following reasons: first, considering the apoenzyme-I complex, the lysine amino group should be close to the 4' carbon atom of the pyridoxamine moiety, and therefore should be unable to react with the position 5 of the dinitrophenyl ring; second, the position of the lysine amino group respective to the coenzy-

TABLE II

Effect of compound I on different Pyridoxal-P-dependent apoenzymes.

Enzyme	Inhibitor concentration (M)	Reaction time (minutes)	pH	Buffer	Inhibition (% of untreated apoenzyme)
Tyrosine aminotransferase	5×10^{-5} M	4	8	0.1 M triethanolamine-HCl	24
Tyrosine aminotransferase	5×10^{-5} M	60	8	0.1 M triethanolamine-HCl	69
Tyrosine decarboxylase	1×10^{-4} M	20	7	0.01 M Tris-HCl	43
Tyrosine decarboxylase	1×10^{-4} M	150	7	0.01 M Tris-HCl	88
Tryptophanase	2.5×10^{-4} M	8	8	0.1 M Phosphate	92
Cystathionase	5×10^{-4} M	60	7.5	0.2 M Tris-HCl	0

The experiments were carried out as described in Table I for aspartate aminotransferase, using for each enzyme the activity assay indicated in the experimental section.

me binding site should be similar for all the apoenzymes so that large differences in reactivity with I are not expected; tables I and II show instead a wide range of reactivity of the different enzymes; in this respect an important observation is that cystathionase binds I only reversibly.

Preliminar spectral studies on the complex between apo-aspartate aminotransferase and I confirm the view that an aminoacid residue different from lysine is responsible for the irreversible inactivation of the apoenzyme.

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