# THE MODE OF INTERACTION OF THE ISONICOTINOYL HYDRAZONE OF PYRIDOXAL PHOSPHATE WITH ASPARTATE-GLUTAMATE APOTRANSAMINASE

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In 1958-1961 several authors (Gonnard, 1958; Gonnard et al., 1959, 1960, 1961; Bonavita a. Scardi, 1959) reported that the isonicotinoyl hydrazone of pyridoxal phosphate (INHPALP), a compound that does not undergo significant hydrolytic dissociation in aqueous solution, will reactivate crude preparations of the apoenzymes of aspartate-glutamate transaminase (AS-transaminase), kynureninase or DOPA-decarboxylase to the same extent as does an equivalent amount of free PALP. Some other hydrazones of PALP similarly exhibit coenzyme activity.

These facts are in apparent contradiction with the inhibitory effects of isoniazid upon transaminases and other PALP enzymes; they also seem to disagree with theoretical considerations indicating that the CO-group of the coenzyme plays an essential part in PALP-dependent enzymatic reactions (cf. Braunstein, 1960). In order to resolve these contradictions we have studied the interactions of INHPALP with the apoenzyme of highly purified AS-transaminase.

INHPALP was synthesized according to Gonnard (1958) with slight modifications. Previously reported methods (Polyanovsky

a. Torchinsky, 1962) were used for the preparation of pig-heart AS-transaminase as the PALP protein (purity grade 95 per cent) and activity assay. Resolution of the AS-transaminase was effected by a modification of the procedure of Wada and Snell (1962); the apoenzyme retained no more than 0.5 per cent of the original AS-transaminase activity and was reactivated to the same extent by equimolar amounts of free PALP or INHPALP. As opposed to INHPALP, the cyanohydrin of PALP was found to produce only a slow and incomplete activation of the apoenzyme.

The absorption spectrum of a solution of INHPALP in 0.05 M acetate buffer (pH 4.6) has a main peak at 295-300 mμ and a smaller peak at 345 mμ (Fig.1, curve 1). Upon addition of a

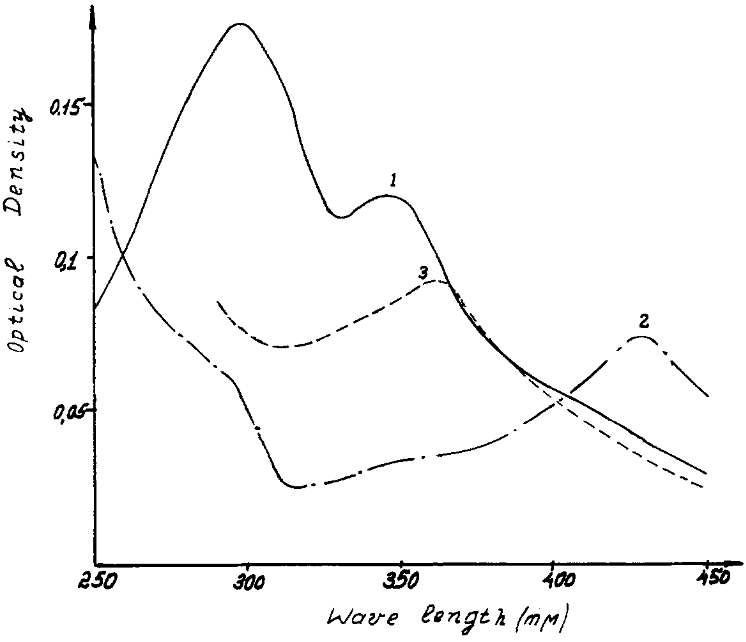

Fig.1. Alterations of absorption spectra on interaction of INHPALP (1.5 mole-equivalents) with AS-apotransaminase (1 mole-equivalent) in 0.05 M acetate buffer (pH 4.6) at 22°.

Curves: 1 - original spectrum of INHPALP solution (3.75 μg/ml); 2 - same solution 30 min after addition of apoenzyme (750 μg/ml), difference spectrum versus pure apoenzyme solution of identical concentration; 3 - same as 2, after alkalinization of sample (to pH 9.0).

solution of AS-apotransaminase to a solution of INHPALP (1.5 mole of the hydrazone per 1 mole of the apoenzyme, mol.w. 110000) there rapidly ensues nearly complete fading of the peaks at 295 and 345 mμ and concomitant appearance of an absorption maximum at 430 mμ (Fig.1, curve 2); these spectral changes run parallel with increase of enzyme activity (Fig.2).

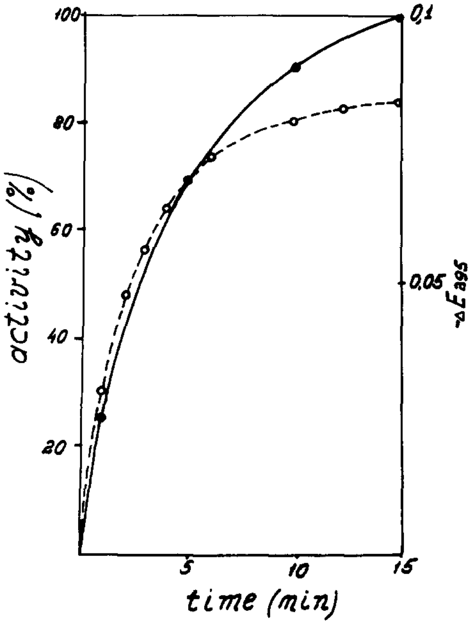

Fig.2. Reactivation of AS-apotransaminase with INHPALP in 0.05 M acetate buffer (pH 5.4) at 23°

Curves: 1.●—● Increase of transaminase activity on incubation of apoenzyme solution (400 μg/ml) with INHPALP (4 μg/ml); 2.○—○ Changes in extinction at 295 mμ ($-\Delta E_{295}$) of the same solution during the incubation.

The absorption maximum at 430 mμ was replaced by a peak at 360 mμ upon alkalinization of the solution (Fig.1, curve 3); after reduction with $NaBH_4$ the maximum was shifted to 330 mμ, with disappearance of transaminase activity.

The spectral and catalytic properties of AS-transaminase reactivated by incubation of the apoenzyme with INHPALP are indistinguishable from those of the original holoenzyme or of the PALP protein reconstituted with the free coenzyme. It is worthy of mention that in similar experiments addition of apotransaminase to a solution of the isonicotinoyl hydrazone of pyridoxal did not cause any changes in the absorption spectrum of the hydrazone.

It is clear from the data reported above that interaction of INHPALP with the apoenzyme results in liberation of PALP and its specific binding to the enzyme protein. It is currently well established that in AS-transaminase and other PALP enzymes the aldehyde group of protein-bound PALP forms an aldimine, or azomethine, bond (intramolecular Schiff base) with the $\varepsilon$-$NH_2$--group of a lysine residue situated at the catalytic site of the apoenzyme (cf. Braunstein, 1960; Turano et al., 1961).

From the results here reported it is evident that the $\varepsilon$-$NH_2$-group of the mentioned lysine residue readily displaces isoniazid from INHPALP bound to the coenzyme-binding sites of AS-apotransaminase, giving rise to the specific intramolecular PALP-aldimine ("transaldiminization" reaction). Thus, INHPALP is not *per se* a coenzyme for apotransaminase as claimed by Gonnard (1961), but rather represents a peculiar artificial precursor of the coenzyme.

Upon addition to a solution of INHPALP of free lysine (in amounts as high as 100-fold molar excess) we failed to observe any significant changes in the absorption spectrum of the hydrazone. This indicates that the $\varepsilon$-$NH_2$-group of the lysine residue located at the catalytic site of AS-apotransaminase has an incomparably higher affinity to the carbonyl group of PALP than has the $\varepsilon$-$NH_2$-group of free lysine. This difference is evidently a result of favorable orientation of the PALP moiety of INHPALP molecules bound at the coenzyme sites of the specific protein, with respect to the $\varepsilon$-$NH_2$-group of the essential lysine residue (positioning effect); no such orientation occurs with the non-phosphorylated hydrazone (vide supra). The high affinity of this $\varepsilon$-amino group to the CO-group of PALP in the native enzyme, due to the steric constraint factor just mentioned, also accounts for the relatively low efficiency of isoniazid and of some other carbonyl reagents when employed as inhibitors of PALP enzymes (Bonavita a. Scardi, 1959; Jenkins et al., 1959; Hicks et al., 1957).

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