PREPARATION OF THE PHOSPHOPYRIDOXAMINE FORM OF THE GLUTAMIC-ASPARTIC TRANSAMINASE*

W. Terry Jenkins and Linda D'Ari

Department of Biochemistry
University of California
Berkeley, California

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The preparation of the phosphopyridoxamine forms of transaminases from their corresponding phosphopyridoxal forms (reaction 1):

amino acid + phosphopyridoxal enzyme $\rightleftharpoons$ keto acid + phosphopyridoxamine enzyme

is complicated by the fact that the overall equilibrium, in those cases which have been studied, markedly favors the phosphopyridoxal form (Jenkins et al., 1960; Velick et al., 1962). To drive the reaction it is thus necessary to remove the keto acid; a variety of methods to accomplish this have been described (Jenkins et al., 1960; Velick et al., 1962; Lis et al., 1960).

The original method of Jenkins and Sizer (1960), in which the mixture of glutamate with the enzyme was passed through a Dowex-1-formate column to remove the ketoglutarate, has been found to suffer from two interrelated drawbacks. If a low buffer concentration is employed, the pH drops, the overall equilibrium becomes even more unfavorable for the production of the phosphopyridoxamine form of the enzyme, and the conversion is incomplete. However, if a high buffer concentration is used, some of the glutamate may not be adsorbed to the column and then contaminates the product. Polyanovskii (1962) has noted that it is necessary to remove this glutamate by Sephadex

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gel filtration after passage of the glutamate-enzyme mixture through the Dowex-1-formate column.

One of the simplest methods to prepare the phosphopyridoxamine form of the enzyme, in principle, is simply to dialyze the enzyme against a solution of amino acid and then remove the excess amino acid by further dialysis (Evangelopoulos et al., 1963).

MATERIALS AND METHODS

Radioactive substrates were obtained from Nuclear Chicago and tested for purity before use. Cysteine sulfinic acid was from the California Corporation, and the other chemicals were also from commercial sources. Only the alpha form of the extramitochondrial pig heart glutamic-aspartic transaminase (EC 2.6.1.1.) was employed in these experiments (Martinez-Carrion et al., 1965). Radioactivity was determined with a Packard Tricarb scintillation counter employing the fluid of Bray (1960).

RESULTS

Dialysis of the glutamic-aspartic transaminase against glutamate and then buffer is effectively accomplished by passage of the enzyme through a long Sephadex, G25 column preceeded by a band of buffered glutamate. The column has to be long enough to give a clean separation between the large and small molecules. If insufficient glutamate is added before the enzyme, the conversion is incomplete. The principle virtues of this method are that, when radioactive glutamate is employed, criteria are established for the degree of small molecule removal and the presence of substrate bound to the protein.

To a 1 x 53 cm column of coarse Sephadex, G25 in 0.05 M sodium pyrophosphate buffer, pH 7.9, was added 2 ml of 10 mM glutamate-^{14}C in the same buffer. This was followed by 1 ml of buffer and then the transaminase (0.1 μmole bound pyridoxal phosphate) in 0.5 ml of buffer containing 10 mM glutamate-^{14}C. Fractions containing 0.8 ml were collected every 0.6 min. upon elution of the enzyme and radioactivity with the original pyrophosphate buffer. If can be seen from Figure 1 that the phosphopyridoxamine form of

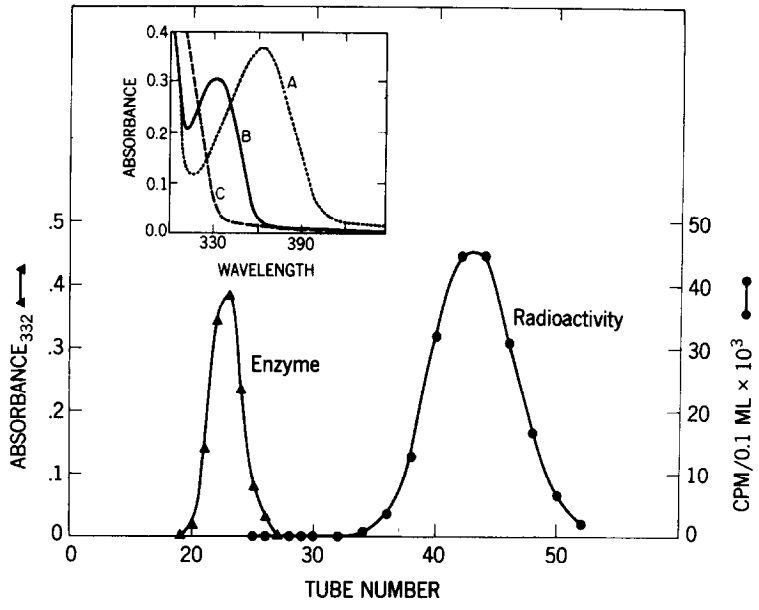

Figure 1

Sephadex dialysis of the pig heart extramitochondrial glutamic-aspartic transaminase against labelled glutamate and then buffer by a procedure decribed in the text. The enzyme, which was eluted from the column by washing with buffer, had been converted into the pyridoxamine form but contained no radioactivity above background. The inset shows the spectrum of a sample of the original enzyme at pH 7.9, and the spectra of the product at pH 7.9 (B) and at pH 13 (C).

the enzyme produced was completely free of labelled substrate. The phosphopyridoxamine enzyme was characterized (inset), in this case, by the fact that it does not yield any pyridoxal phosphate (λmax 388 mμ) upon treatment with alkali.

Lis and colleagues (1960) reacted the phosphopyridoxal form of this enzyme with aspartate and subsequently removed the oxalacetate by reduction with malic dehydrogenase. We confirmed that this is a suitable procedure and that if radioactive aspartate is employed, the product contains no radioactivity after passage through a Sephadex column.

The most convenient method we have found for the preparation of the phosphopyridoxamine form of this enzyme, however, is to react it with cysteine sulfinate. Upon the addition of cysteine sulfinate to the phos-

phopyridoxal form of the enzyme, three successive reactions may be observed spectroscopically. First, there is an instantaneous spectral change due to the establishment of the amino acid-keto acid equilibrium. This is followed by a further rapid (1 min.) change in the absorbance to yield an amount of the phosphopyridoxamine form equivalent to the concentration of cysteine sulfinate employed. This phase is due to the decomposition of the sulfinylpyruvate produced to SO_2 and pyruvate (Leinweber et al., 1962). The last slow phase of the reaction is due to the pyruvate reacting with the phosphopyridoxamine form of the enzyme to regenerate the phosphopyridoxal form (Jenkins, 1961). The enzyme may readily be retained in the phosphopyridoxamine form either by reducing the pyruvate with lactic dehydrogenase or more simply by the addition of an excess of the cysteine sulfinate.

We make the phosphopyridoxamine form of the enzyme routinely by

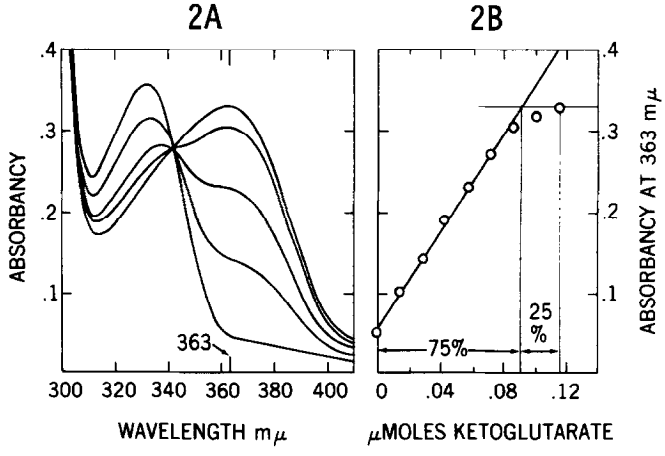

Figure 2A

Spectrophotometric titration of a sample of the phosphopyridoxamine form of the glutamic-aspartic transaminase with 8 successive 2 μl aliquots of 7.22 mM α-ketogluterate-U-^{14}C (50 μ curies/ml).

Figure 2B

The data of part 2A are plotted to show the stoichiometry over the early part of the titration. The basis for the estimation of a 25% excess of ketogluterate added in this experiment is indicated.

the addition of a slight excess of cysteine sulfinate followed immediately by removal of the small molecules through Sephadex dialysis. We have never noted any spontaneous decomposition of the product back to the phosphopyridoxal form.

Figure 2 shows that the phosphopyridoxamine form of this enzyme (λmax 332 mμ) can be titrated back to the pyridoxal form by the addition of successive aliquots of radioactive ketoglutarate. The conversion is equal to the amount of ketoglutarate added up to about 60% reaction when the concentration of glutamate formed in the reaction becomes appreciable. From the graph (Figure 2B) we estimated that, in this particular experiment, the final conversion of the enzyme was about 92% completed, but that only 75% of the ketoglutarate had reacted. The enzyme was subsequently separated from the small molecules with the same Sephadex column as before, and the extent of conversion of the enzyme confirmed by the determination of pyridoxal phosphate upon the addition of alkali. All of the radioactivity was associated with the small molecules, and, of this, 67% was found to be glutamate and 33% α-ketoglutarate by the method of Waksman and Roberts (1965).

Reaction with mixtures of substrates

When mixtures of erythro-β-hydroxy-L-aspartate and glutamate are added to the phosphopyridoxal form of the glutamic-aspartic transaminase, the resultant spectrum is markedly different from those in the presence of equivalent concentrations of either amino acid alone (Jenkins, 1964).

Recently, we have carefully reinvestigated the nature of these spectral changes quantitatively in order to detect possible 'ternary complexes' containing both glutamate and hydroxyaspartate (Jenkins, et al., submitted for publication). It was found that whereas the spectra were not dependent upon the order of addition of the two amino acids, they were very sensitive indeed to the concentration of enzyme employed. This dependency upon the concentration of enzyme indicates the involvement of reactions of the phosphopyridoxamine form. In brief, it was found that

the observed changes could be completely accounted for by the formation of only binary complexes. The marked decrease in absorbance at 492 mμ upon the addition of glutamate last was shown to be due, for the most part, to the reaction:

glutamate + pyridoxal enzyme - hydroxyaspartate $\rightleftharpoons$

ketoglutarate + pyridoxamine enzyme - hydroxyaspartate

The increase in absorbance at 492 mμ when hydroxyaspartate is added last was due mostly to the overall reaction:

pyridoxamine enzyme + ketoglutarate + hydroxyaspartate $\rightleftharpoons$

pyridoxal enzyme - hydroxyaspartate + glutamate

DISCUSSION

Evangelopoulos and Sizer (1963) stated 'that there is no evidence for a pyridoxamine transaminase'. This conclusion was based principally on their claim (Evangelopoulos et al., 1963, 1965) that the pyridoxal form was completely regenerated upon total removal of the amino acid substrate from the enzyme. As shown in this paper, our attempt to confirm this finding by Sephadex gel filtration was not successful. The suggestion by these same workers (Evangelopoulos et al., 1965) that the form of the enzyme which yields pyridoxamine phosphate upon treatment with alkali as prepared by the Dowex-1-formate procedure also contains firmly bound glutamate, appears to be without experimental justification (Polyanovskii, 1962) since no attempts either to remove it by dialysis or even show that the glutamate was, in fact, closely associated with the enzyme as it came from the column were reported.

Although the phosphopyridoxamine form of the enzyme absorbs at 332 mμ, this spectral maximum is typical of very many Vitamin B_6 derivatives. The absence of an absorption maximum at 388 mμ in 0.1N alkali together with the reactivity with keto acids are much better criteria for characterization than the absorption maximum alone.

We believe that the pyridoxal-pyridoxamine hypothesis has satisfactorily withstood all experimental tests of its validity (Jenkins, 1963).

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