Glutamic-Aspartic Transaminase

REACTION OF HOLOENZYME WITH SUBSTRATES AND OF APOENZYME WITH VITAMIN B₆ DERIVATIVES*  

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The general subject of enzymatic transamination has been extensively reviewed by several workers (Braunstein (1); Snell (2); Meister (3)). Glutamic-aspartic transaminase (l-aspartate:2-oxoglutarate aminotransferase, EC 2.6.1.1) from pig heart has been prepared by Jenkins, Yphantis, and Sizer (4) and shown to contain 2 molecules of pyridoxal phosphate per enzyme molecule of molecular weight of about 116,000. Under special conditions the enzyme can be resolved and reconstituted by the addition of pyridoxal phosphate (5-7). In the case of reactivation with pyridoxamine phosphate some workers obtained none, while others found a retarded reactivation (8-10).

The reaction of dicarboxylic keto acid substrates to the enzyme, which has a maximum absorption at 362 μm, results in the formation of a complex with a maximum in the region of 350 μm which the carboxyl groups are reversibly bound to the enzyme (4, 11). Amino substrates also react with the enzyme as indicated by the shift of the maximum absorption peak to 333 μm (11, 12). This change produced by amino substrates has been interpreted by Jenkins and Sizer (12) and Lis et al. (13) as indicating the formation of a pyridoxamine-P enzyme-amino acid complex. The possibility that an enzyme-amino acid complex was an intermediate in the formation of pyridoxamine-P acid complex was studied later from the enzyme on a Dowex 1-formate column (11, 14). This change was produced from radioactive glutamate by the method of Wada and Snell (6) and from transaminase to which had been added 0.1 μm glutamate or aspartate (the excess amino substrate was separated later from the enzyme on a Dowex 1-formate column, and the enzyme finally resolved by the same method); (c) from transaminase plus aspartate or glutamate by the method of Scardi et al. (7). All calculations of apoenzyme molarity were based on a molecular weight of 120,000. In all cases the apoenzyme was freed from trace amounts of possible contaminants, such as keto acids, by extensive dialysis or passage through a Dowex 1-formate column. When radioactive α-ketoglutarate or glutamate was added to the enzyme, the apoenzyme isolated according to Wada and Snell (6) was not radioactive. L-Glutamate, L-aspartate, L-glutamate-14C, α-ketoglutarate-5-14C, DL-threo-β-hydroxyaspartate, DL-threo-β-hydroxyaspartate, pyruvate, glyoxylate, α-ketobutyrate, α-ketovalerate, α-ketoacaproate, α-ketoisovalerate, α-ketoisocaprate, formaldehyde, acetaldehyde, acetone, glyceraldehyde, pyridoxal-P, pyridoxamine-P, pyridoxine-P, 4-deoxypyridoxine-P, pyridoxamine hydrochloride, and pyridoxal hydrochloride were supplied by the California Corporation for Biochemical Research.

**Methods**

Oxaloacetate, α-ketoglutarate, and other keto acids were determined as their hydrazones by the method of Friedemann and Haugen as modified by Koepall and Sharp (15). This procedure is sensitive to about 0.01 to 0.005 μmole of total keto acid. To 0.5 ml of the solution containing the enzyme, buffer, and the corresponding amino acid (after standing for 5 min at 25°), 1 ml of 10% metaphosphoric acid (prepared fresh each time) was added to precipitate the protein. The protein was then removed by centrifugation. Keto acid in the supernatant solution was determined as its hydrazone after addition of spectral characteristics.

In the present paper an attempt is made to obtain further information on the mechanism of transamination by a measurement of the reaction of transaminase with amino substrates to produce enzyme-substrate complex, pyridoxamine-P enzyme, and keto acids. We have also studied the interaction of several vitamin B₆ derivatives with apotransaminase in order to correlate spectral changes with restoration of enzymatic activity produced by pyridoxal-P and pyridoxamine-P.

**EXPERIMENTAL PROCEDURE**

**Materials**

The glutamic-aspartic transaminase from pig heart was prepared by the method of Jenkins et al. (4) and was dialyzed against pyridoxal-P followed by dialysis against water. The enzyme preparations used in all the experiments had an absorption ratio (A_{362}/A_{280}) between 9.5 and 10.5 and a specific activity of 32 units (4). Glutamic-aspartic apotransaminase was prepared in three different ways: (a) from the enzyme by the method of Wada and Snell (6); (b) from transaminase to which had been added 0.1 μm glutamate or aspartate (the excess amino substrate was separated later from the enzyme on a Dowex 1-formate column, and the enzyme finally resolved by the same method); (c) from transaminase plus aspartate or glutamate by the method of Scardi et al. (7). All calculations of apoenzyme molarity were based on a molecular weight of 120,000. In all cases the apoenzyme was freed from trace amounts of possible contaminants, such as keto acids, by extensive dialysis or passage through a Dowex 1-formate column. When radioactive α-ketoglutarate or glutamate was added to the enzyme, the apoenzyme isolated according to Wada and Snell (6) was not radioactive. L-Glutamate, L-aspartate, L-glutamate-14C, α-ketoglutarate-5-14C, DL-threo-β-hydroxyaspartate, DL-threo-β-hydroxyaspartate, pyruvate, glyoxylate, α-ketobutyrate, α-ketovalerate, α-ketoacaproate, α-ketoisovalerate, α-ketoisocaprate, formaldehyde, acetaldehyde, acetone, glyceraldehyde, pyridoxal-P, pyridoxamine-P, pyridoxine-P, 4-deoxypyridoxine-P, pyridoxamine hydrochloride, and pyridoxal hydrochloride were supplied by the California Corporation for Biochemical Research.

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Chromatographic identification of radioactive α-ketoglutarate produced from radioactive glutamate was achieved by the method of Bush and Hockaday (10). The radioactive glut-
Transaminase activity was measured by oxaloacetate formed from L-aspartate and a-ketoglutarate by the method of Tonhazy et al. as modified by Wada and Snell (6). A quantity of the enzyme was assayed by transferring it to a 3-ml reaction mixture containing 50 μmoles of aspartate, 50 μmoles of a-ketoglutarate, and 100 μmoles of ethylenediaminetetraacetic acid in 0.05 M phosphate buffer, pH 7.4. Transaminase activity is expressed as micrograms of oxaloacetate produced in 10 min at 37°C. Standard curves with oxaloacetate were calculated, and it was carried as a standard in every determination of enzymatic activity. Protein was determined by the method of Lowry et al. (19).

RESULTS

1. Keto Acid Production

Evangelopoulos and Sizer (11), using as their index the change in spectrum of transaminase produced by keto acids, were unable to find any keto acid production upon the addition of stoichiometric amounts of amino acid to enzyme. Jenkins and Sizer (12) and Lis et al. (13) reported the production of appreciable amounts of keto acid when relatively large amounts of amino substrates were added to the enzyme.

In the present investigation we have studied keto acid production by the dinitrophenylhydrazine method (15) at concentrations of amino substrates from 2 × 10⁻⁴ to 2 M in 1 × 10⁻⁴ M transaminase (calculated on the basis of a purity of 80% and a molecular weight of 120,000). Results at pH 9.0, 5.5, and 4.2 for glutamate and aspartate are shown in Fig. 1 and Table II. The system contained different concentrations of transaminase in 0.02 M carbonate, pH 9.0. Keto acid production was measured after addition of glutamate in a final concentration of 0.2 M and in a final volume of 0.5 ml.

![Fig. 1. Ketoglutarate or oxaloacetate production resulting from the reaction of increasing amounts of glutamate or aspartate with enzyme at pH 9.0 (+), 5.5 (☆), and 4.2 (□) was measured.](http://www.jbc.org/)

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Concentration</th>
<th>α-Ketoglutarate or oxaloacetate produced*</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Glutamate</td>
<td>2 × 10⁻⁴</td>
<td>0.73 (µg) 0.48 (µg) 0.41 (µg)</td>
</tr>
<tr>
<td>L-Aspartate</td>
<td>2 × 10⁻⁴</td>
<td>0.06 (µg) 0.45 (µg)</td>
</tr>
</tbody>
</table>

*α-Ketoglutarate was produced from L-glutamate, and oxaloacetate from L-aspartate.

<table>
<thead>
<tr>
<th>Transaminase</th>
<th>μg</th>
<th>α-Ketoglutarate</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.16</td>
<td>0.057</td>
<td>35.5</td>
</tr>
<tr>
<td>0.08</td>
<td>0.029</td>
<td>35</td>
</tr>
<tr>
<td>0.06</td>
<td>0.019</td>
<td>37.5</td>
</tr>
<tr>
<td>0.01</td>
<td>0.0075</td>
<td>37.5</td>
</tr>
</tbody>
</table>

* Calculation based as in Fig. 1 on the assumption that both pyridoxal-P groups in transaminase are available for reaction with glutamate. If only one group is involved in transamination the figures in the third column should be doubled.

In spectrum of transaminase at 333 nm is produced (11), there is about 0.005 μmole of keto acid formed. Maximal spectral
change is produced by $2 \times 10^{-2} \text{ M}$ aspartate or glutamate, while maximum keto acid production is not reached at a concentration of $2 \text{ M}$ substrate. Keto acid is produced very rapidly upon the addition of amino substrates to enzyme and is complete in 1 min. Keto acid production per mole of enzyme is independent of enzyme concentration from $1 \times 10^{-5}$ to $2 \times 10^{-4} \text{ M}$ (see Table II). The amount of keto acid produced is about the same at pH 9.0 and 4.2 (Fig. 1, Table I) whereas the enzyme activity is maximal at pH 9.0 and essentially zero at pH 4.2. From this study of keto acid production it appears that the spectral changes produced by the addition of amino substrates to the enzyme are not correlated with the formation of a comparable amount of keto acids. In addition, keto acid production from glutamate or aspartate does not appear to require conditions suitable for catalysis of the over-all transamination reaction.

2. Studies with Labeled Substrates

14C-Glutamate—Another approach to the study of the interaction of the enzyme and an amino substrate is to investigate the transaminase system to which had been added 14C-glutamate. After the enzyme had reacted with substrate for 15 min at 25°, the system was examined for production of radioactive ketoglutarate and radioactive enzyme-glutamate complex.

A system of $1 \times 10^{-4} \text{ M}$ transaminase in 0.1 M 14C-glutamate at pH 8.5 was placed on a column of Dowex 1-formate or of Dowex 1-formate after equilibration of the column with borate or Tris buffer, and the radioactivity of transaminase after elution with buffer was measured. In four separate experiments the enzyme was found to be radioactive; the amount of radioactivity corresponded to that which would be expected if 1 to 2 molecules of 14C-glutamate were combined with 1 molecule of enzyme (Table III).

In order to determine the possible formation of radioactive α-ketoglutarate in a similar experiment, the Dowex column was subjected to gradient elution with 1 N HCl. The first component to be eluted is the unchanged radioactive glutamate which is separated from the second component, radioactive ketoglutarate (identified as ketoglutarate by paper chromatography). In a typical experiment 200 μmoles of glutamate with a radioactivity of 675,000 cpm were added to 0.83 μmole of transaminase in 2 ml of 0.02 M borate buffer, pH 8.5. After passage through a Dowex column the radioactivity was distributed as follows: enzyme, 5,500 cpm; glutamate, 647,000 cpm; ketoglutarate, 1,750 cpm; recovery of radioactivity, 97%. The amount of ketoglutarate recovered in this experiment and others as measured chemically and by radioactivity is roughly 30% of the theoretical amount and is comparable to that shown in Fig. 1 for 0.1 M glutamate.

14C-Ketoglutarate—The possibility that amino acid might be produced from keto acid plus pyridoxamine-P enzyme was investigated with the use of radioactive ketoglutarate plus apoenzyme (prepared according to Wada and Snell (6)) plus pyridoxamine-P. In a typical experiment 2 μmoles of pyridoxamine P were added to a solution of 0.65 μmole of apotransaminase in 2 ml of 0.02 M borate buffer, pH 9.0, at 37°. After 2 hours of incubation 2 μmoles of α-ketoglutarate characterized by a radioactivity of 68,200 cpm were added. After incubation for 10 min the system was passed through a Dowex borate column which was subjected to gradient elution with 1 N HCl. The radioactivity was distributed as follows: enzyme, 218 cpm; α-ketoglutarate, 53,792 cpm; glutamate, 10,842 cpm; recovery of radioactivity, 94%. The glutamate produced was identified chromatographically and measured both with ninhydrin and by radioactivity. It was found to be about 20% of the theoretical amount expected from 0.65 μmole of apoenzyme. In other experiments under the same conditions but with 0.02 M Tris buffer, pH 8.5, 37% of the theoretical yield of glutamate was recovered.

3. Studies with β-Hydroxyaspartate

The reaction of amino substrates with transaminase is indicated by the shift in absorption peak of the spectrum from 362 μm at pH 8.5, which is characteristic of amino acid alone, to 333 μm, which is characteristic of enzyme plus amino substrate. The spectrum of enzyme plus glutamate is shown in Fig. 2. Although there is no agreement (11–13) about the interpretation of the peak at 333 μm, Evangelopoulos and Sizer (11) have shown that it reverts to 362 μm upon removal of the amino substrate by dialysis. Jenkinson has indicated that erythro-β-hydroxyaspartate reacts with the pyridoxal-P enzyme to give a complex with a sharp peak at 492 μm (20). It is thus possible, after the addition of erythro-β-hydroxyaspartate, to use the absorption at this wavelength as a measure of the amount of pyridoxal-P enzyme present in the system containing enzyme plus glutamate.

If $4 \times 10^{-2} \text{ M}$ erythro-β-hydroxyaspartate is added to transaminase in $4 \times 10^{-2} \text{ M}$ glutamate at pH 8.5, the characteristic 333 μm maximum disappears and is replaced by the maximum at 492 μm characteristic of the pyridoxal-P enzyme-erythro-β-hydroxyaspartate complex (Fig. 2). Similar results are obtained with aspartate at several concentrations. These data suggest that in the system containing transaminase plus glutamate or aspartate the enzyme is predominantly in the pyridoxal-P form, in agreement with the conclusions reached on the basis of keto acid production. Since erythro-β-hydroxyaspartate has a very high affinity for the active site, the enzyme is bound preferentially by it instead of by the glutamate or aspartate.

Three-β-Hydroxyaspartate reacts with transaminase and produces an absorption peak at 333 μm (typical of enzyme plus amino substrate) in sharp contrast to the peak at 492 μm produced by erythro-β-hydroxyaspartate (20). It was therefore of interest to compare keto acid production in both systems with the use of $1 \times 10^{-2}$ and $1 \times 10^{-1} \text{ M}$ concentrations of these hydroxy acids. In neither system over this concentration range was any keto acid detected under the experimental con-
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ditions normally employed for glutamate and aspartate at pH 9.0 in the presence of $1 \times 10^{-4} \text{ M}$ transaminase.

4. Reaction of Apotransaminase with Pyridoxal-P and Pyridoxal

When apotransaminase is prepared by any of the three methods described in “Experimental Procedure,” it has an activity less than 1% and is characterized by the absorption spectrum shown in Fig. 3. To the apoenzyme solution was added a stoichiometric amount of a vitamin B$_6$ derivative. In each case the system was studied with reference to spectrum and transaminase activity before and after dialysis. Because of the sharply distinct spectra of the free vitamin derivatives and because a stoichiometric amount of apoenzyme absorbs only slightly in this region of the spectrum, it is possible to interpret the results unambiguously. The addition of pyridoxal-P to apoenzyme prepared by any of the three methods restored activity (see Section 5, “Pyridoxamine-P,” and Fig. 7) and the native spectrum of transaminase (Fig. 3). The absorption maxima changed from 330 $\mu$m and 388 $\mu$m, characteristic of free pyridoxal-P (21), to a new maximum at 362 $\mu$m, characteristic of the holoenzyme (4). The identical experiment with pyridoxal produced an inactive complex characterized by a spectrum strikingly different from that produced with pyridoxal-P (Fig. 3). In the case of free pyridoxal the absorption maxima occur at 318 $\mu$m and 390 $\mu$m (21) whereas the apoenzyme-pyridoxal complex has a maximum at 410 $\mu$m (uncorrected for the slight effect of a small amount of free pyridoxal in the system).

In order to gain further understanding of the differences in the interaction of pyridoxal-P and pyridoxal with apotransaminase we have studied changes in spectrum as a function of the amount of these vitamin B$_6$ derivatives added to the system. Strikingly different results obtained for pyridoxal-P and pyridoxal are shown in Fig. 4. In the case of pyridoxal-P the absorption at 360 $\mu$m (maximum for the holoenzyme at pH 8.5) increases until a plateau is reached at a concentration of 2 molecules of pyridoxal-P per molecule of enzyme. In a somewhat similar manner the maximum at 410 $\mu$m for pyridoxal plus apotransaminase increases with pyridoxal concentration, although the reaction does not appear to be stoichiometric. If the absorption of the pyridoxal-P-apoenzyme is examined at 410 $\mu$m, it changes only slightly during the titration until the stoichiometric concentration, which produces the 360 $\mu$m plateau, is reached. Titration with pyridoxal-P beyond this concentration causes the 410 $\mu$m curve to rise abruptly in a manner comparable to the curve produced by pyridoxal at the same wavelength (Fig. 4). It thus appears that pyridoxal-P below its stoichiometric concentration can bind apotransaminase specifically ($\lambda_{\text{max}} = 360 \mu$m) while above this concentration it behaves like pyridoxal and binds to apoenzyme nonspecifically ($\lambda_{\text{max}} = 410 \mu$m). Unlike pyridoxal-P, pyridoxal does not bind to produce a unique product with absorption at 360 $\mu$m (Figs. 3 and 4).

In view of the remarkable effects of pH changes on the spectrum of transaminase (4), it was of interest to examine the effect of lowering the pH to 5.5 (0.02 M phosphate buffer) upon the
reaction between apotransaminase and pyridoxal-P or pyridoxal. Since transaminase at this pH has absorption maxima at 430 and 340 m\(\mu\) (11), we have used the absorption at 430 m\(\mu\) as an index of interaction between apoenzyme and pyridoxal-P or pyridoxal (Fig. 5). Reconstitution as a function of pyridoxal-P concentration occurs at pH 5.5 in a manner very similar to pH 8.5 as shown by the increase in absorption at 430 m\(\mu\). On the other hand, no evidence of any reaction between pyridoxal and apotransaminase is obtained from studies at different wave lengths at pH 5.5 (Fig. 5).

Pyridoxal-P is firmly bound to apoenzyme, and the enzyme formed is stable to dialysis; hence the specific binding of pyridoxal-P to apotransaminase as measured by absorption at 360 m\(\mu\) at pH 8.5 does not change upon dialysis (Table IV). On the other hand, the nonspecific binding observed at high pyridoxal-P concentrations and measured by absorption at 410 m\(\mu\) is readily reversed by dialysis. Pyridoxal at all concentrations is bound to apotransaminase to give a product with an absorption peak at 410 m\(\mu\). Dialysis of this product for 1 hour results in a rapid release of apotransaminase plus free pyridoxal (Table IV).

**Fig. 4 (upper).** Changes in the absorption at 360 and 410 m\(\mu\) observed during titration by pyridoxal-P or pyridoxal of the apoenzyme. The reaction mixture consisted of 0.06 \(\mu\) mole of apotransaminase in 0.3 ml of 0.02 M phosphate buffer, pH 5.5, plus pyridoxal-P or pyridoxal.

**Fig. 5 (lower).** Changes in the absorption at 430 m\(\mu\) observed during the titration with pyridoxal P or pyridoxal of the apoenzyme at pH 5.5. The reaction mixture consisted of 0.06 \(\mu\) mole of apotransaminase in 0.3 ml of 0.02 M phosphate buffer, pH 5.5, plus pyridoxal-P or pyridoxal.

### Table IV

<table>
<thead>
<tr>
<th>Vitamin B, (21)</th>
<th>Absorption maxima after reaction with transaminase</th>
<th>Product after dialysis for 4 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyridoxal</td>
<td>318, 390</td>
<td>410</td>
</tr>
<tr>
<td>Pyridoxal-P</td>
<td>330, 388</td>
<td>362</td>
</tr>
<tr>
<td>Pyridoxamine</td>
<td>325</td>
<td>325</td>
</tr>
<tr>
<td>Pyridoxamine-P</td>
<td>325</td>
<td>330-340</td>
</tr>
<tr>
<td>Pyridoxine-P</td>
<td>325</td>
<td>325</td>
</tr>
<tr>
<td>Deoxypyridoxine-P</td>
<td>315</td>
<td>315</td>
</tr>
</tbody>
</table>

* *One hour of dialysis is enough for complete separation.
† The original maximum at 325 m\(\mu\) of pyridoxamine-P when apotransaminase forms a complex with pyridoxamine-P gradually changes to higher wave lengths with time. Dialysis of the solution results in accumulation of pyridoxal-P enzyme as described in the text.

5. Reaction of Apotransaminase with Pyridoxamine-P, Pyridoxamine, Pyridoxine-P, and 4-Deoxypyridoxine-P

In view of the different behavior of pyridoxamine-P as compared with pyridoxal-P in the activation of apotransaminase (8–10) we have studied this system with the use of pyridoxamine-P, pyridoxamine, pyridoxine-P, and 4-deoxypyridoxine-P. As criteria for interaction between vitamin derivative and apoenzyme we have measured changes in spectrum, enzyme activity, and other properties before and after dialysis.

**Pyridoxamine-P**—When pyridoxamine-P is added to apotransaminase under a variety of conditions of temperature and pH, the spectrum of the system gradually changes from that of free pyridoxamine-P to a spectrum which indicates the production of the pyridoxal-P enzyme. The absorption maximum at 325 m\(\mu\) (characteristic of pyridoxamine-P at pH 7.0 (21)) gradually decreases and changes to 330 to 340 m\(\mu\), while the absorption at 362 m\(\mu\) (characteristic maximum of transaminase above pH 7.0) and 436 m\(\mu\) (characteristic maximum of transaminase below pH 7.0 (4)) gradually increases (Fig. 6). In this system conversion to the pyridoxal-P enzyme is incomplete, but conversion is complete when the system is dialyzed against water (Table IV) and proceeds more rapidly when the system is dialyzed against adipate, maleate, erythro-\(\beta\)-hydroxyaspartate, or phosphate (0.02 M, pH 5.5). The spectral changes which occur when pyridoxamine-P is added to apotransaminase are unique to this system. This is shown by the fact that when pyridoxamine-P is added to a solution of albumin under the same conditions there is no change in the spectrum, which remains that of pyridoxamine-P.

When pyridoxal-P or pyridoxamine-P is added to apotransaminase, the activity is restored to that of the original enzyme in the manner shown in Fig. 7. At pH 7.4 (0.05 M phosphate buffer) the reactivation with pyridoxamine-P proceeds much more slowly than with pyridoxal-P. We have also studied this system in carbonate buffer (0.05 M, pH 9.0) and in phosphate buffer (0.05 M, pH 5.5) and found, as compared with pH 7.4, a greater difference between pyridoxal-P and pyridoxamine-P at pH 5.5, and a smaller difference at pH 9.0.
Fig. 6 (upper). Spectral changes at 325 m\(\mu\) (absorption maximum of pyridoxamine-P) and 362 and 426 m\(\mu\) (absorption maxima of transaminase) which occur during the reaction of apotransaminase and pyridoxamine-P. The reacting system contained 0.05 \(\mu\)mole of apotransaminase and 0.1 \(\mu\)mole of pyridoxamine-P in 0.3 ml of 0.02 M phosphate buffer, pH 6.5.

Fig. 7 (lower). Restoration of enzyme activity by preincubation at 37° of apotransaminase with pyridoxal-P or pyridoxamine-P. Apotransaminase (1 mg) was incubated with 0.05 \(\mu\)mole of pyridoxal-P or pyridoxamine-P in 5 ml of 0.05 M phosphate buffer, pH 7.4. At the periods indicated in the figure, 10 \(\mu\)g of the enzyme were assayed by transferring it to a 3-ml reaction mixture containing 50 \(\mu\)mole of aspartate, 50 \(\mu\)mole of \(\alpha\)-ketoglutarate, and 100 \(\mu\)mole of EDTA in 0.05 M phosphate, pH 7.4. Transaminase activity is expressed as micrograms of oxaloacetate produced after a 10-min incubation at 37°. Oxaloacetate formed from L-aspartate and \(\alpha\)-ketoglutarate was measured by the method of Tonhazy et al. as modified by Wada and Snell (6).
The increase in activity after addition of pyridoxamine-P to apotransaminase might bear some relationship to the changes in spectrum shown in Fig. 6. A possible interpretation for this relationship is that the pyridoxamine-P apotransaminase initially formed is converted to active pyridoxal-P enzyme.

During the study of spectral changes upon the addition of pyridoxamine-P to apotransaminase we observed a retardation of the spontaneous formation of pyridoxal-P enzyme produced by the addition of amino substrates. In order to determine whether or not these amino substrates were also interfering with the restoration of activity, we have studied the influence of pre-incubation of the apoenzyme with aspartate from 10^-4 to 10^-1 M upon both the apotransaminase-pyridoxal-P and the apotransaminase-pyridoxamine-P systems (Fig. 8). Whereas the restoration of the pyridoxal-P enzyme is independent of aspartate concentration below 10^-3 M, for pyridoxamine-P the restoration is inhibited at all concentrations which have been used. This study shows that inhibition by aspartate of the recombination of apotransaminase plus pyridoxamine-P to transaminase as indicated by the delay in spectral changes is correlated with the delay in the production of active enzyme.

Since the previous experiments suggest that the restoration of activity by the addition of pyridoxamine-P to apotransaminase might involve the spontaneous formation of pyridoxal-P enzyme, we have attempted to measure pyridoxal-P enzyme in another way. The pyridoxal-P enzyme when combined with erythro-β-hydroxyaspartate is characterized by a very sharp absorption peak at 492 nm (20). It is therefore possible to use the absorption at this wavelength as a direct measure of the concentration of pyridoxal-P enzyme in a solution containing pyridoxamine-P, apotransaminase, and erythro-β-hydroxyaspartate.

Typical experiments at 37° with 0.2 μmole of pyridoxal-P or 0.05 μmole of apotransaminase in 0.5 ml of carbonate buffer (0.05 M, pH 9.0) or in phosphate buffer (0.05 M, pH 7.4) containing 2 × 10^-3 M erythro-β-hydroxyaspartate were presented in Fig. 9, in which absorption at 492 nm is plotted as a function of time. The reaction is highly sensitive to pH over the range from pH 5.5 to 10.5, with an optimum in the vicinity of pH 9.0. Similarly, the rate increases rapidly with temperature from 25° to 50° and decreases above 60°. The reaction proceeds more rapidly as the relative concentration of pyridoxamine-P is increased and becomes maximal when twice the stoichiometric amount of pyridoxamine-P is used. Inhibition occurs when pyridoxamine-P is 20 times the stoichiometric concentration. Studies on pH, temperature, and pyridoxamine-P concentration are all consistent with the suggestion that the production of pyridoxal-P enzyme, after the addition of pyridoxamine-P to apotransaminase, is enzyme-catalyzed. Dialysis of the complex with the absorption maximum at 492 nm formed in the system containing apotransaminase, pyridoxamine-P, pyridoxal-P-hydroxyaspartate results in the accumulation of pyridoxal-P enzyme in the system.

The nature of the spontaneous slow transformation of pyridoxamine-P-apotransaminase to pyridoxal-P enzyme is not known. Since many workers (22-24) have demonstrated the conversion of free pyridoxamine-P to pyridoxal-P by an oxidative deamination brought about by crude enzymes from different sources, it was of interest to determine whether or not our transaminase system was similar, as indicated by a dependence upon oxygen of the spontaneous transformation. In three experiments the system contained 0.08 μmole of apotransaminase and 0.8 μmole of pyridoxal-P in 3 ml of 0.1 M carbonate buffer, pH 9.0, which contained 0.1 M erythro-β-hydroxyaspartate at 37°. One system was studied in air, another in nitrogen after evacuation of air, and the third in oxygen after bubbling oxygen through the solution. Results shown in Fig. 10 suggest a direct or indirect dependence upon oxygen of the spontaneous transformation. In three experiments the system contained 0.05 μmole of apotransaminase and 0.8 μmole of pyridoxal-P in 3 ml of 0.1 M carbonate buffer, pH 9.0, which contained 0.1 M erythro-β-hydroxyaspartate at 37°. One system was studied in air, another in nitrogen after evacuation of air, and the third in oxygen after bubbling oxygen through the solution. Results shown in Fig. 10 suggest a direct or indirect dependence upon oxygen of the spontaneous transformation of pyridoxal-P-apotransaminase in pyridoxal-P enzyme. Attempts to demonstrate by the Conway technique and Nessler's reagent the production of ammonia during the incubation of pyridoxal-P and apotransaminase were unsuccessful. The rate of transformation is not affected by the presence of 10^-6 M ethylenediaminetetraacetic acid.

**Pyridoxamine-P plus Keto Acids**—In Section 2 it was shown that the addition of keto acid substrate to the equilibrated system containing pyridoxamine-P plus apotransaminase results in the formation of amino acid (12, 13). Since the spontaneous conversion of pyridoxamine-P apoenzyme to pyridoxal-P enzyme seems to be dependent on oxygen, it was of interest to perform an experiment under nitrogen with the use of pyridoxa-
p-hydroxyaspartate was measured in air, under nitrogen after mine-P in 3 ml of 0.1 M carbonate buffer, pH 9.0, and 0.01 M erythro-enzyme-hydroxyaspartate complex, is measured as a function of time. The optical density of 492 nm of the reacting system completely to pyridoxal-P enzyme. Conversion by the first three compounds is rapid and of the same magnitude, whereas with α-ketobutyrate and glyoxylate the conversion is slower (see Table VI).

Among other keto acids tested, α-ketovalerate, α-ketocaproate, and α-ketoisocaproate also bring about the formation of pyridoxal-P enzyme and behave like α-ketobutyrate and glyoxylate. Of all the keto acids tested, α-ketobutyrate convert apoenzyme plus pyridoxamine-P completely to pyridoxal-P enzyme. Conversion by the first three compounds is rapid and of the same magnitude, whereas with α-ketobutyrate and glyoxylate the conversion is slower (see Table VI).

Other Vitamin B₆ Derivatives—Further insight into the reaction of apotransaminase with pyridoxal-P and pyridoxamine-P can be obtained by investigating the reaction of the apoenzyme with other vitamin B₆ derivatives such as pyridoxamine, pyridoxine-P, and 4-deoxypyridoxine-P. Pyridoxine-P combines with apotransaminase to produce a complex with a spectrum which does not change with time. The complex with pyridoxal-P has the same peak at 325 nm as free pyridoxine at the same pH (Table IV). This complex is relatively stable and can be dialyzed for 4 days at 4° before apotransaminase is released in appreciable amounts. The phosphorylated derivative, 4-deoxypyridoxine-P, reacts with apotransaminase to produce a characteristic complex with spectral properties similar to that of 4-deoxypyridoxine-P at the same pH (Table IV). This complex behaves like that with pyridoxine-P when dialyzed.

The nonphosphorylated derivative of pyridoxal-P does not undergo any change in spectrum upon addition of apotransaminase and, unlike pyridoxamine-P, the spectrum of the system does not change with time. The pyridoxamine was rapidly removed from the system by dialysis (Table IV).
It is apparent from these studies with vitamin B₆ derivatives that the presence of a phosphate group is important in the binding of the derivative to apotransaminase. In the absence of a phosphate group an amino derivative (pyridoxamine) does not undergo any change after it becomes attached to apotransaminase. When both phosphate and amino groups are present (pyridoxamine-P), strong binding to apotransaminase occurs; this is followed by a slow reaction which leads to the formation of a mixture of pyridoxamine-P apoenzyme and pyridoxal-P enzyme.

**DISCUSSION**

Changes in absorption spectrum produced by the addition of amino substrates to transaminase occur at low substrate concentration (11), while production of keto acid becomes appreciable only at high amino substrate concentration (see Table I, Fig. 1) (12, 13). The production of keto acid upon addition of amino substrates to transaminase might suggest that this is a crucial step in the mechanism of transamination. However, the possibility that keto acid production is an artifact is not excluded since high concentrations of amino substrates are required (Table I, Fig. 1). Of equal interest is the fact that the reverse reaction can be shown by the use of ¹⁴C-α-ketoglutarate apoenzyme plus pyridoxamine-P. In this system the production of pyridoxal-P enzyme plus ¹⁴C-glutamate can be demonstrated, although the data obtained using a Dowex column are somewhat qualitative. These data on aerobic and anaerobic production of pyridoxal-P enzyme plus glutamate from pyridoxamine-P and apoenzyme plus ketoglutarate are of course consistent with the pyridoxal-pyridoxamine hypothesis of transamination.

Inconsistent with the idea of the participation of the above two half-reactions in the mechanism of transamination is the observation that keto acid production from the reaction of transaminase with amino substrates is nearly independent of pH from 4.2 to 9.0 (Table I, Fig. 1), while the rate of enzymatic transamination changes from zero to maximum over the same range of pH. The recovery from a Dowex column of a radioactive enzyme which binds nearly stoichiometrically amounts of amino substrate (Table III) raises the possibility that such a complex might play a role in transamination. The absorption spectrum (333 mm maximum) of enzyme plus amino substrates has been interpreted as indicating the formation of the pyridoxamine-P enzyme. Our experiments suggest that a pyridoxal-P enzyme-amino acid complex is mainly responsible for this peak. Both phosphorylase (26) and glutamic acid decarboxylase (27) are characterized by a peak at 333 mm, yet there is no evidence for the presence of pyridoxamine-P in these enzymes. The observation that erythro-β-hydroxyaspartate reacts with the glutamate-enzyme system absorbing at 333 mm to produce a pyridoxal-P enzyme-erythro-β-hydroxyaspartate complex (492 mm maximum, Fig. 2) suggests that an appreciable amount of pyridoxal-P enzyme is present in the system containing glutamate. An alternative explanation is that an amino substrate such as erythro-β-hydroxyaspartate, which might be expected to react with the pyridoxal-P enzyme to convert it to pyridoxamine-P enzyme, behaves in the opposite way by converting the pyridoxamine-P enzyme (333 mm form) to the pyridoxal-P enzyme (Fig. 2).

A different type of complex is obtained from the reaction of the α-isomer of hydroxyaspartate (28). The absorption spectrum (410 mm maximum) of enzyme plus amino substrate, produces the 333 mm peak with transaminase yet is a very poor substrate (20). Erythro-β-hydroxyaspartate, unlike a typical amino substrate, produces a 492 mm peak (instead of 333 mm) with the enzyme, yet it is a very much better substrate than the three isomer (20). The 333 mm form of the enzyme with three-β-hydroxyaspartate cannot indicate the presence of pyridoxamine-P enzyme because no detectable keto acid was produced by the reaction of either of these isomers of hydroxyaspartic acid with the enzyme. Whatever end products result from interaction of enzyme and amino substrates as evidenced by the peak at 333 mm, it is clear that the system is reversible, since dialysis results in the formation of pyridoxal-P enzyme which absorbs at 362 mm (11).

Information concerning the binding of pyridoxal-P to the active center of transaminase has been obtained from the studies of apotransaminase plus pyridoxal or pyridoxal-P. It is well known that the aldehyde group of pyridoxal and pyridoxal-P reacts at neutral pH with most amino acids and with serum albumin to form Schiff bases with spectral absorption maxima at about 410 mm (28-31). Similarly, pyridoxal reacts with apotransaminase to form a complex which absorbs at 410 mm. On the other hand, pyridoxal-P reacts specifically with the active center of the apotransaminase to form the holoenzyme which absorbs at 362 mm. Above a concentration in excess of 2 molecules of pyridoxal-P per enzyme molecule, the excess pyridoxal-P combines with apotransaminase to form a complex which absorbs at 410 mm like that formed by pyridoxal (Fig. 4). It thus appears that the phosphate group of pyridoxal-P plays a critical role in binding (1, 2) and contributes also to the final configuration of the coenzyme in the active center as shown by the enzyme spectrum (Fig. 3). Several workers have suggested that the interaction between apoenzyme and pyridoxal-P involves in part the formation of a Schiff base between pyridoxal-P and the ε-amino group of lysine (32-34). Since this complex with pyridoxal-P, which appears to involve binding by both phosphate and aldehyde groups, is stable to dialysis (in contrast to the pyridoxal complex), it has properties different from a prosthetic group held by a Schiff base linkage (Fig. 3) (11, 25). The difference between pyridoxal-P and pyridoxal is further emphasized by studies of their reaction with apotransaminase at pH 5.5. Pyridoxal does not form a detectable Schiff base (29) at this pH, while pyridoxal-P reacts to produce the usual pyridoxal-P enzyme (Fig. 5).

The gradual restoration of transaminase activity (Fig. 7) upon the addition of pyridoxamine-P to apotransaminase appears to be correlated with the formation of pyridoxal-P enzyme (Figs. 6 and 9). This formation of pyridoxal-P enzyme may be enzymatic as suggested by the effects of pH, temperature, pyridoxamine-P concentration, and inhibitors upon the rate, although these variables may affect metal ion catalysis in a similar way. The dependence upon oxygen of the spontaneous transformation of pyridoxamine-P plus apoenzyme to the pyridoxal-P enzyme (Fig. 10) shows that oxidation is involved. Although ammonia formation could not be demonstrated, there remains the possibility that it was produced but trapped in the system. Further insight into this reaction is given by studies on other vitamin B₆ derivatives. The spontaneous formation of pyridoxal-P enzyme requires the presence of both phosphate and amino groups in the vitamin molecule which is added to apoenzyme. This conclusion is supported by studies which have been made with pyridoxamine, pyrioxine-P, and 4-deoxypyridoxine-P, which behave differently from pyridoxamine-P. Since a wide variety of keto acids can rapidly convert the pyridoxamine-P apoenzyme to pyridoxal-P enzyme (Table VI), the
possibility exists that the slow spontaneous conversion might involve a reaction between pyridoxamine-P and a carbonyl group of the apoenzyme. This suggestion is consistent with the finding that the amino group of pyridoxamine-P is no longer available for acetylation after its reaction with apotransaminase (36).

The turnover number (moles of product formed per mole of enzyme per min) of the reaction between α-keto acids and pyridoxamine-P catalyzed by apotransaminase (without preincubation of apotransaminase with pyridoxamine-P) (Table VI) is about 30. This figure is 1000 times smaller than the turnover of transamination catalyzed by the holoenzyme, and it is of the same magnitude as the rate of the reaction between pyridoxamine and α-keto substrate catalyzed by apoenzyme previously reported (9). This reaction of pyridoxamine-P plus keto acid plus apoenzyme is much faster than the spontaneous transformation in the absence of added keto acid.

During the last 10 years, the pyridoxal-pyridoxamine hypothesis of enzymatic transamination, with a Schiff base as an intermediate, has been accepted by most investigators. In addition, a Schiff base has been postulated to play a role in other pyridoxal P catalyzed reactions. In a previous communication we have criticized this hypothesis partly because stoichiometric amounts of amino substrates markedly changed the enzyme spectrum in a reversible manner without the formation of stoichiometric amounts of keto substrates. In the present paper, however, we have found that keto acid production becomes appreciable at high amino substrate concentration (Table 1, Fig 1). We assume that a comparable amount of pyridoxamine-P enzyme is also produced. Difficult to interpret by the pyridoxal-pyridoxamine hypothesis is the fact that the production of keto acid is much less than stoichiometric even at 2 M aspartate or glutamate concentration; this system is insensitive to pH and is reversible upon dialysis. In contrast the reverse half-reaction (pyridoxamine-P apoenzyme with α-keto acids) has a wide specificity, yields pyridoxal-P enzyme even with stoichiometric amounts of α-keto acids (Table VI), and is not reversed by dialysis.

Other evidence which we have raised (11) against the pyridoxal-pyridoxamine hypothesis does not directly disprove it. The fact that keto substrates and amino substrates form reversible, spectroscopically distinct complexes with transaminases does not prove their involvement in a ternary mechanism. Similarly the demonstration that certain inhibitors mimic keto substrates and others amino substrates in their reaction with transaminase can be explained by either the binary or ternary hypothesis. Our inability to isolate pyridoxamine-P after resolution of transaminase under conditions of equilibrium with aspartate (11) is due to the fact that the isolation of pyridoxamine-P enzyme requires very special procedures (12, 13). Spectral changes of the enzyme produced by substrates, inhibitors, and change in pH are now interpreted in terms of effects on the configuration of the vitamin B6 in the active site, and their interpretation need not exclude the binary hypothesis.

While data presented in the previous (11) and present paper are consistent with a ternary scheme for transamination, they do not disprove the pyridoxal-pyridoxamine hypothesis. Certain questions are raised, however, the answers to which will eventually lead to a better understanding of transamination. Several postulations for the mechanism of transamination seem possible (37-45) and should be further investigated.

**Summary**

1. Keto acid is produced when aspartate or glutamate is added to glutamic-aspartic transaminase. At stoichiometric concentrations, keto acid is hardly detectable, while at 2 M it becomes 35% (aspartate) or 50% (glutamate) of the theoretical amount. This production is not pH-dependent from pH 4.2 to 9.0.

2. Studies with 14C-labeled glutamate indicate that a radioactive complex with transaminase is formed which contains 1 to 2 molecules of glutamate per molecule of enzyme. Radioactive ketoglutarate is also produced in this system. Radioactive glutamate is formed in the reverse reaction with pyridoxamine-P, apoenzyme, and 14C-α-ketoglutarate.

3. Cytathr-β-Hydroxyaspartate, which forms a colored complex with pyridoxal phosphate transaminase, has been used to demonstrate the presence of pyridoxal phosphate enzyme in a system which contains glutamate plus enzyme.

4. Pyridoxal phosphate and pyridoxal combine rapidly but very differently with glutamic-aspartic apotransaminase. Two molecules of pyridoxal phosphate combine firmly with 1 molecule of apoenzyme to produce holoenzyme with full activity and a characteristic absorption peak at 362 mp. Pyridoxal combines loosely and nonstoichiometrically with apoenzyme to produce an inept complex with an absorption peak at 410 mp. Above its stoichiometric concentration, pyridoxal phosphate reacting with apotransaminase also produces an absorption peak at 410 mp.

5. Pyridoxamine phosphate combines with apotransaminase to produce a complex which is slowly transformed to a mixture of pyridoxamine-P apoenzyme and pyridoxal-P enzyme. This transformation is dependent on pyridoxamine phosphate concentration, proceeding much more slowly at low pH and temperature, and is inhibited by amino substrates. No transformation occurs if pyridoxamine is used in place of pyridoxamine phosphate. A variety of α-keto acids participate in a specific transamination of pyridoxamine-P by the apotransaminase to produce pyridoxal-P enzyme.

6. All phosphorylated derivatives of vitamin B6 which have been studied bind firmly to apotransaminase and cannot be separated by dialysis at all, or only after 4 days. On the other hand, the nonphosphorylated derivatives which have been studied bind loosely to apotransaminase and can be removed by dialysis for 1 hour.

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**References**


Glutamic-Aspartic Transaminase: REACTION OF HOLOENZYME WITH SUBSTRATES AND OF APOENZYME WITH VITAMIN B6 DERIVATIVES
A. E. Evangelopoulos and Irwin W. Sizer


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