The Interaction of Cycloserine with Glutamate Aspartate Transaminase as Measured by Fluorescence Spectroscopy*

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SUMMARY

The reaction of cycloserine with pyridoxal 5-phosphate has been investigated by fluorescence spectroscopy. The substantial change in fluorescence intensity at 450 nm that follows the reaction of cycloserine with pyridoxal 5-phosphate was used to monitor the release of the cofactor from the active site of the enzyme glutamate aspartate transaminase. A kinetic analysis of the fluorescence measurements reveals that the resolution of the enzyme is favored at pH values below 5 in the presence of phosphate ions. The inactive species produced by the reaction with cycloserine can be reactivated by incubation with pyridoxal 5-phosphate. These studies provide information on the firmness of binding of pyridoxal 5-phosphate to the active site of the enzyme.

It was shown by Khomutov, Karpeisky, and Severin (1) that the reaction of the racemate of the antibiotic cycloserine with the enzyme glutamate aspartate transaminase results in complete inhibition of the enzymatic activity. One of the interesting features of this reaction is that the enzyme-cycloserine complex is stable and can be characterized by spectrophotometric methods. According to Karpiysk and Breusov (2), the change in the absorption spectrum can be related to the formation of pyridoxal 5-phosphate-cycloserine Schiff base since the reaction of cycloserine with free pyridoxal 5-phosphate causes similar spectral changes.

It is shown in the present paper that cycloserine reacts with the enzyme glutamate aspartate transaminase to produce characteristic changes in the fluorescent properties of the bound cofactor. These changes in the fluorescent properties, particularly the increase in the intensity of fluorescence at 450 nm, were used to monitor the release of pyridoxal 5-phosphate from the active site of the enzyme.

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EXPERIMENTAL PROCEDURE

Materials

Glutamate aspartate transaminase, which was purchased from C. F. Boehringer (Control No. 6356215), was purified by hydroxylapatite chromatography according to the method of Jenkins, Yphantis, and Sizer (3). This preparation had a specific activity of 30 units per mg of protein and exhibited a symmetrical absorption maximum at 362 nm, pH 8.5, which indicated that the enzyme was in the pyridoxal 5-phosphate form. The pyridoxal 5-phosphate content of this preparation (1.8 moles/110,000 mol wt) was determined by the procedure of Wada and Snell (4). Hydroxylapatite (Bio-Gel HTP Control No. 4323) was purchased from Bio-Rad Laboratories, cycloserine (o-4-amino-3-isoxazololone) from Nutritional Biochemicals, and pyridoxal 5-phosphate from Sigma. All the chemical reagents were of analytical grade.

Methods

Preparation of Material—Prior to the resolution experiments, samples of enzyme dissolved in 0.02 M sodium phosphate buffer, pH 6.80, were brought to the desired pH by dialysis against 0.1 M sodium phosphate buffers. The sample of enzyme at pH 4.3 was obtained by dialyzing against several changes of 0.1 M NaH₂PO₄ at 4° for 6 hours. In the resolution experiments, the enzyme, at a concentration of 0.4 mg of protein per ml, was allowed to react with excess cycloserine (10⁻² M) for 1 hour at 30°.

Enzymatic activity was determined in 0.1 M Tris-acetate buffer, pH 8.2, at 30° by the increase in optical density at 280 nm due to oxalacetate formation in the transaminase reaction between α-ketoglutarate and aspartate, according to the method of Sizer and Jenkins (5). A Zeiss spectrophotometer was used in these studies. Protein concentrations were determined by the procedure of Lowry et al. (6).

Fluorescence Measurements—Fluorescence emission spectra were obtained with the use of a spectrofluorimeter designed in our laboratory. The sample in a 1-cm cuvette was illuminated by monochromatic light obtained by passing the output of a xenon lamp (Hanovia, 150 watt) through a Bausch and Lomb...
monochromator (linear dispersion, 6.6 μm per mm). The light emitted at right angles to the exciting light source was passed through a second Bausch and Lomb monochromator (linear dispersion, 6.6 μm per mm). An EMI phototube (6256S) attached to a recording photometer (model 15, Pacific Instruments) was used as the fluorescence detector. Calibration of the exciting light source was carried out with solutions of rhodamine B in ethyleneglycol or 1-dimethylaminonaphthalene 5-sulphonate in 0.1 M NaHCO₃ as fluorescence screens by the method of Melhuish (7). The detector system was calibrated according to the method of White, Ho, and Weimer (8). A bandwidth of 2 μm was used in the fluorometric determinations. Quantum yields of fluorescence (Q) were calculated according to the method of Parker and Rees (9) with standards of known quantum yields (fluoresceine, quinine sulphate, and dimethylaminonaphthalene sulphonate) (10, 11).

RESULTS

The reaction of pyridoxal 5-phosphate with cycloserine is a particularly convenient one for study for two reasons. First, the reaction proceeds to completion at a readily measurable rate, and second, the fluorescence changes that occur upon pyridoxal 5-phosphate-cycloserine Schiff base formation facilitate measurement of the course of the reaction. As shown in Fig. 1, the reaction of cycloserine with pyridoxal 5-phosphate occurs over a wide pH range and can be detected by a marked increase in the fluorescence intensity at 450 nm. The reaction is favored at pH values lower than 5 and approaches completion only when the cycloserine is present in large excess (10⁻² M). It should be noted that the results shown in Fig. 1 were obtained at pyridoxal 5-phosphate concentrations lower than 10⁻³ M, since the fluorescence emitted by the Schiff base is easily detected in the spectrofluorimeter. The change in fluorescence as a function of time is adequately represented by the expression

\[ \log (F_t - F_d + k_{ob} \cdot t + \text{constant}) \]

where \( k_{ob} \) is the observed first order rate constant for Schiff base formation, \( F_M \) the maximum intensity of fluorescence at completion of the reaction, and \( F_t \) the intensity of fluorescence at time \( t \). The rate constants calculated for the reactions conducted at different pH values are included in Fig. 2 and Table I.

From these results it is evident that the rate of reaction between pyridoxal 5-phosphate and cycloserine can be increased by as much as three-fold by decreasing the pH of the medium. In addition, it is noteworthy that the quantum yield of fluorescence of the Schiff base is several orders of magnitude larger than the corresponding value of free pyridoxal-5-phosphate (Q = 0.01) (Table I).

The emission spectrum of the pyridoxal 5-phosphate-cycloserine Schiff base remains virtually unchanged from about pH 4.3 to pH 8, showing one maximum of emission at 455 nm (Fig. 3).

Inactivation of Glutamate Aspartate Transaminase—The fluorometric method described in the preceding section may be used conveniently to monitor the interaction of the inhibitor cycloserine with pyridoxal 5-phosphate residues which are bound to the active site of the glutamate aspartate transaminase enzyme (12). To this end a large excess of cycloserine was added to the enzyme solution (0.5 mg per ml) and the course of the reaction was followed by two independent methods: fluorescence and activity measurements. For fluorescence measurements, a blank cuvette which contained the same concentration of pyridoxal 5-phosphate and cycloserine as the enzyme solution was used as standard. For activity measurements, aliquot samples were removed from the reaction mixture at different time intervals and assayed for enzymatic activity (5). At pH 4.3, the reaction between cycloserine and the holoenzyme was completed in 1 hour, as judged by a 30-fold increase in the fluorescence.
TABLE I
Reactions of $3.3 \times 10^{-6}$ M pyridoxal 5-phosphate with $10^{-2}$ M cycloserine at $80^\circ$C

<table>
<thead>
<tr>
<th>pH</th>
<th>Observed first order rate constant</th>
<th>Quantum yield of fluorescence</th>
<th>Fluorescence maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\text{min}^{-1}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.3</td>
<td>0.20</td>
<td>0.21</td>
<td>455</td>
</tr>
<tr>
<td>4.7</td>
<td>0.18</td>
<td>0.21</td>
<td>455</td>
</tr>
<tr>
<td>5.0</td>
<td>0.16</td>
<td>0.20</td>
<td>455</td>
</tr>
<tr>
<td>6.0</td>
<td>0.10</td>
<td>0.20</td>
<td>455</td>
</tr>
<tr>
<td>7.2</td>
<td>0.06</td>
<td>0.20</td>
<td>455</td>
</tr>
</tbody>
</table>

Fig. 3. Corrected emission spectra for the pyridoxal 5-phosphate-cycloserine Schiff base obtained at pH 4.3 (○) and pH 7.2 (△). The optical density of the samples at the exciting wavelength (350 mp) was 0.1 for 1-cm cuvettes. Areas beneath the curves are proportional to the fluorescence quantum yield.

The course of the reaction could be described by Equation 1 and the rate constant for fluorescence enhancement was 0.020 min$^{-1}$ (Fig. 2). When the reaction between cycloserine and the pyridoxal 5-phosphate residues of the aspartate transaminase enzyme was monitored by activity measurements, it was found that the loss of activity as a function of time of incubation followed first order kinetics; therefore the rate constant ($k = 0.073 \text{ min}^{-1}$) was determined by plotting the logarithm of the specific activity versus time (Fig. 4). A comparison between the rate constants ($k = 0.020 \text{ min}^{-1}$ and $k = 0.073$) corresponding to fluorescence enhancement and enzyme inactivation, respectively, reveals that the latter process proceeds faster than the increase in fluorescence intensity. This lack of parallelism between the two processes suggests that the binding of cycloserine to the holoenzyme results in immediate loss of activity and that the increase in fluorescence at 450 mp occurs only after the release of the pyridoxal 5-phosphate-cycloserine Schiff base from the active site of the enzyme. Additional support for the contention that the pyridoxal 5-phosphate-cycloserine Schiff base detached from the enzyme surface is responsible for the increase in the fluorescence intensity was provided by inhibition experiments conducted at neutral pH (7.2).

When the cycloserine-aspartate transaminase reaction was carried out in neutral solution (pH 7.2), the rapid decrease in the enzymatic activity was still observed, but the subsequent increase in the fluorescence intensity was so slow as to be nearly negligible (Fig. 1). As shown in Table II, the enzyme-cycloserine complex proved to be enzymatically inactive regardless

of the pH at which the reaction was performed. Furthermore, the failure of the complex to show a substantial increase in the fluorescence intensity at neutral pH, such as has been observed at acid pH, suggests that the pyridoxal 5-phosphate-cycloserine Schiff base is bound to the protein. The binding of the Schiff base to acceptor sites on the enzyme was found to be greatly affected by the pH and composition of the medium. This is shown in Table II, from which it can be seen that the resolution of the enzyme-cycloserine complex depends upon the acidity of the solution. From these results it is also evident that the inactive enzyme-cycloserine complex, prepared at neutral pH, recovers a good deal of its activity (70%) after dialysis against 0.1 M Tris-acetate buffer, pH 8.2. This finding is in sharp contrast to the behavior of the inactive species prepared at acid pH.

<table>
<thead>
<tr>
<th>Experimental conditions for inactivation with $10^{-4}$ M cycloserine</th>
<th>Rate constant$^*$</th>
<th>Inactivation by $10^{-4}$ M pyridoxal 5-phosphate</th>
<th>Dialysis against 0.1 M Tris-acetate buffer, pH 8.2</th>
<th>Reconstitution with $10^{-4}$ M pyridoxal 5-phosphate</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 M NaH$_2$PO$_4$, pH 4.3</td>
<td>0.073</td>
<td>3</td>
<td>10</td>
<td>90</td>
</tr>
<tr>
<td>Phosphate buffer, pH 4.7</td>
<td>0.073</td>
<td>4</td>
<td>15</td>
<td>95</td>
</tr>
<tr>
<td>Phosphate buffer, pH 6.0</td>
<td>0.070</td>
<td>5</td>
<td>50</td>
<td>95</td>
</tr>
<tr>
<td>Phosphate buffer, pH 7.2</td>
<td>0.068</td>
<td>5</td>
<td>70</td>
<td>95</td>
</tr>
</tbody>
</table>

* First order rate constant for inactivation.
pH, which, according to the results summarized in Table II, displayed low residual activity after dialysis against 0.1 M Tris-acetate buffer, pH 8.2. All of these observations are compatible with the hypothesis that the fluorescence changes which follow the inhibition of the enzyme glutamate aspartate transaminase by cycloserine are related to the release of the pyridoxal 5-phosphate-cycloserine Schiff base from the active site of the enzyme. In this context, the increase in the fluorescence intensity at 450 nm reflects the removal of the pyridoxal 5-phosphate-cycloserine Schiff base from the enzyme surface.

Reconstitution of Aspartate Transaminase—In view of the results described in the preceding section, it was of interest to determine whether the enzyme treated with cycloserine could be reactivated by addition of pyridoxal 5-phosphate. Previous to the reconstitution experiments, the inactive species treated with cycloserine were dialyzed against several changes of 0.1 M Tris-acetate buffer, pH 8.2, at 4°C. The reconstitution mixture, which contained 0.3 mg of protein per ml and 10^{-5} M pyridoxal 5-phosphate, was incubated at 30°C for 1 hour. It was then diluted with Tris-acetate buffer, pH 8.2, and assayed for activity according to the method of Sizer and Jenkins (5). The results of the reconstitution studies, performed under the experimental conditions described above, indicate that approximately 90% recovery of the original activity was achieved after 1 hour of incubation at 30°C. In confirmation of earlier work on the reconstitution of this enzyme (13-15), it was found that the rate at which restoration of activity occurs is affected by the pH and the temperature of the system.

**DISCUSSION**

The fluorescence changes produced by the addition of cycloserine to glutamate aspartate transaminase can be used to the release of pyridoxal 5-phosphate from the active site of the enzyme. An essential point to be considered in the resolution studies is that the experimental conditions chosen must be sufficient to insure the cleavage of the covalent and electrostatic bonds which link the cofactor to the enzyme. Thus, it was found that at acid pH, in the presence of phosphate ions, the reaction of cycloserine with the enzyme results in a substantial loss of enzymatic activity even after dialysis against Tris buffer, pH 8.2. At a pH closer to neutral, however, the same series of reactions failed to resolve the enzyme to any great extent. In all cases, however, the inactive species could be reactivated by addition of pyridoxal 5-phosphate, and a recovery of approximately 90% of the original activity was achieved under appropriate conditions. Although the reaction of cycloserine with the enzyme was followed by two independent methods, fluorescence and activity measurements, it was observed that there is no close correlation between changes in fluorescence intensity and loss of enzymatic activity. The most clear-cut difference between the two processes is that the loss of activity proceeded faster than the increase in fluorescence intensity at 450 nm, as indicated by the fact that the rate constants are of a different order of magnitude.

It is proposed that the lack of parallelism between the two measurements can be explained in terms of the firmness of binding of the pyridoxal 5-phosphate-cycloserine Schiff base to the enzyme. The following theory is suggested for the interpretation of the experimental results.

1. The internal Schiff base between pyridoxal 5-phosphate and lysine groups of the enzyme is disrupted by cycloserine. As a result of this disruption, the enzymatic activity is lost.

2. The increase in fluorescence intensity at 450 nm that follows the inhibition of the enzyme is due to the release of the pyridoxal 5-phosphate-cycloserine Schiff base from the active site of the enzyme.

3. Any delay in the enhancement of fluorescence is attributed to electrostatic interactions which hinder the release of the Schiff base.

The experimental observations on the inhibition of the enzyme at neutral pH fit very well into the theory developed above. It is possible that the fluorometric method described in this work can provide useful information on the binding of pyridoxal 5-phosphate to the active site of other transaminases since it is well established that cycloserine is a strong inhibitor of several enzymes of this group (16, 17).

**REFERENCES**


The Interaction of Cycloserine with Glutamate Aspartate Transaminase as Measured by Fluorescence Spectroscopy
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