RESOLUTION AND RECONSTITUTION OF GLUTAMATE DECARBOXYLASE FROM CEREBELLUM

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Brain glutamate decarboxylase (EC 4.1.1.15) catalyzes the biosynthesis of the postulated neurotransmitter γ-aminobutyric acid according to the following chemical equation: L-glutamate → γ-aminobutyric acid + CO₂. Hydroxylamine treatment of the decarboxylase at low ionic strength followed by Sephadex gel filtration resolves apoenzyme from cofactor (>90%). Pyridoxal phosphate completely restores activity. Sodium borohydride inactivates the holoenzyme, but not the apoenzyme. This supports the notion that pyridoxal phosphate is bound to the holoenzyme as a Schiff base. Moreover, salicylaldehyde, a reagent which reacts with amino groups, substantially inactivates the apoenzyme but not the holoenzyme. Reconstitution of the bovine cerebellar holoenzyme from apogluta-
mate decarboxylase and pyridoxal phosphate occurs in seconds to minutes, which is much faster than that of the decarboxylase isolated from E. coli. Native holoenzyme, apoenzyme, and reconstituted holoenzyme have identical molecular weights as estimated by Sephadex gel filtration.

INTRODUCTION

γ-Aminobutyric acid (GABA) is an established inhibitory neurotransmitter at the crustacean neuromuscular junction and a probable neurotransmitter in the vertebrate central nervous system. The GABA concentration in brain is in the millimolar range, and 30% of all the synaptosomes (pinched-off nerve endings) prepared from rat brain

1 A preliminary account of this work has been presented (1).
sequester labeled GABA by a high-affinity uptake system (2). Although GABA has been considered primarily an inhibitory neurotransmitter in the central nervous system, recent evidence suggests a possible excitatory role. Our previous study, for example, has shown that the olfactory nerve of the gar fish (Lepisosteus osseus) and pike (Esox estor) contains GABA and its biosynthetic enzyme (3). Moreover, Flock and Lam (4) have reported similar results for fish inner ear sense organs and lateral line organs, and sensory hair cells of amphibians.

L-Glutamate decarboxylase (EC 4.1.1.15) catalyzes GABA biosynthesis according to the following reaction: l-glutamate $\rightarrow$ GABA + CO$_2$. Several lines of evidence support the idea that pyridoxal phosphate is the cofactor for this reaction. For example, pyridoxal phosphate stimulates enzyme activity in vitro (5) and hydrazides (and other carbonyl-trapping reagents) inhibit activity in vitro and in vivo (6). Moreover, decarboxylase activity in vitro is decreased in rats with vitamin-B$_6$ deficiency. These results, however, fail to directly demonstrate that pyridoxal phosphate is the enzyme cofactor. Sheep liver serine dehydratase, for example, is inhibited by carbonyl-trapping reagents and binds one mole of pyridoxal phosphate per 20,000 g of protein (7). Recent studies by Kapke and Davis, however, show that electrophoretically homogeneous serine dehydratase lacks pyridoxal phosphate and contains α-ketobutyrate as the carbonyl cofactor (8).

We report on the resolution of apoglutamate decarboxylase from cofactor and the reconstitution of activity by the addition of pyridoxal phosphate.

**EXPERIMENTAL PROCEDURE**

*Materials.* Uniformly labeled L-[14C]glutamic acid was purchased from New England Nuclear Co. Sodium borohydride was obtained from Fisher Scientific. Dithiothreitol was from RSA Corporation, ultrapure ammonium sulfate from Schwartz-Mann, and salicylaldehyde from Aldrich Chemical Co. DEAE-cellulose (DE-52) was purchased from Reev Angel. Pyridoxal phosphate and its congeners, aminoethylisothiourea bromide (AET), hydroxylamine sulfate, and Sephadex gels were obtained from Sigma Chemical Co.

*Substrate Purification.* Commercial L-[14C]glutamic acid was purified by ascending paper (Whatman No.1) chromatography in 5:4:1 n-butanol-water-acetic acid. Purified substrate ($R_f = 0.3$) was resolved from an interfering contaminant ($R_f = 0.95$) and eluted from the air-dried chromatogram with 0.01 M HCl. Recoveries were 80–85% based on radioactivity.

*Glutamate Decarboxylase Assay.* Enzyme activity was determined by measuring the formation of γ-[U-14C]aminobutyrate from L-[U-14C]glutamate. Specific activity was measured by incubating a 15-μl aliquot of enzyme solution with 15 μl of a solution containing 5 mM L-glutamate (0.19 μCi), 0.1 M potassium phosphate, and 0.1 mM
pyridoxal phosphate (pH 7.4) at 37°C for 10 min unless specified otherwise. After
terminating the reaction with 3 μl of 0.5 N formic acid (containing 20 mM γ-aminobutyrate),
product was resolved from substrate by paper (Whatman No. 1) electrophoresis in
8% acetic acid-29% formic acid for 20 min at 40 V cm⁻¹ (ambient temperature).
Radiolabeled product was quantitated as previously described (9). Specific activity is
expressed as nmols γ-aminobutyrate min⁻¹ mg⁻¹ protein.

Holoenzyme Purification. Glutamate decarboxylase was purified by modifications of the
method of Susz et al. (10). Modifications included: (a) the source was bovine cerebellum;
(b) DEAE-cellulose column chromatography was performed using 20 mM morpholinopro-
pane sulfonic acid (MOPS) buffer (pH 7.0) in place of phosphate buffer, and a linear
potassium chloride gradient from 0 to 0.5 M to elute the enzyme; and (c) Sephadex G-200
gel filtration was carried out with 50 mM MOPS buffer, pH 7.0. The holoenzyme obtained
was stored at 4°C in 0.1 M potassium phosphate, 1 mM AET, and 0.2 mM pyridoxal
phosphate (pH 7.4) at a concentration of 40-50 mg protein/ml using the method of Lowry
et al. (11). This enzyme extract (specific activity range of 4-10) was used as needed for the
preparation of apoenzyme. Fresh holoenzyme preparations were made regularly at 2-week
intervals.

Preparation of Apoglutamate Decarboxylase. Holoenzyme extract (0.5 ml) was dialyzed
12 hr at 4°C against 1000 volumes of 20 mM potassium phosphate, 1 mM AET (pH 7.4). The
dialed enzyme solution was then mixed with 0.5 ml of solution containing 0.1 M
hydroxylamine sulfate, 2 mM AET, 0.2 mM dithiothreitol, and 50% glycerol (w/v)
(adjusted to pH 7.4 with KOH), and incubated for 10 min at 37°C. The preparation was then

![Graph](image)

**Fig. 1.** Molecular weight of apo- and hologlutamate decarboxylase. Molecular weights
were estimated by Sephadex G-200 chromatography (1.3 × 105-cm column). Human γ-
globulin (150,000) (12), bovine serum albumin (69,000) (13), ovalbumin (44,000) (14), and
myoglobin (17,200) (15) were used as molecular-weight markers. Potassium phosphate (0.1
M, pH 7.4) containing 0.1 mM dithiothreitol was used to elute the proteins; 0.1 mM
pyridoxal phosphate was included in experiments with holoenzymes. The elution volume
(Vₑ) was taken as the position of maximal enzyme activity. V₀ refers to the void volume.
### TABLE I

**Resolution of Glutamate Decarboxylase**

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Pyridoxal phosphate (0.6 mM)</th>
<th>Specific activity</th>
<th>Resolution (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Holoenzyme extract</td>
<td>+</td>
<td>4.09</td>
<td>0</td>
</tr>
<tr>
<td>Dialysis&quot;</td>
<td>-</td>
<td>3.72</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>4.09</td>
<td></td>
</tr>
<tr>
<td>Final product</td>
<td>-</td>
<td>0.38</td>
<td>91</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>4.09</td>
<td></td>
</tr>
</tbody>
</table>

" Results shown are typical of most preparations, although some variation does occur. Enzyme activity yields are generally 90-100%, and the resolution achieved is usually 90-98%.

" Assays performed as described in Experimental Procedure.

" Refers to holoenzyme extract dialyzed 12 hr against 20 mM potassium phosphate, 1 mM AET, pH 7.4.

### TABLE II

**Effect of Sodium Borohydride Treatment on Glutamate Decarboxylase**

<table>
<thead>
<tr>
<th>pH</th>
<th>Pyridoxal phosphate</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.4</td>
<td>-</td>
<td>2.8</td>
</tr>
<tr>
<td>7.4</td>
<td>+</td>
<td>1.4</td>
</tr>
<tr>
<td>6.3</td>
<td>-</td>
<td>3.7</td>
</tr>
<tr>
<td>6.3</td>
<td>+</td>
<td>0.44</td>
</tr>
</tbody>
</table>

" To apoglutamate decarboxylase (2.4 mg protein in 300 µl buffer A, specific activity 3.9 in the presence of 0.1 mM pyridoxal phosphate, prior to treatment) was added 10 µl of 10 mM pyridoxal phosphate (+) or H₂O₂ (−) and incubated 5 min at 37°. Octanol (1 µl) and 0.5% (w/v) NaBH₄ (50 µl) were then added, and the solution was incubated on ice for 20 min. The solutions were dialyzed against 800 volumes of buffer A for 4 hr at 4° with one change of buffer. The preparations were assayed for enzyme activity as described in Experimental Procedure with 0.1 mM pyridoxal phosphate.

" Prepared by dialysis against buffer A adjusted to pH 6.3.
passed through a Sephadex G-25 column (1.3 x 24 cm) at ambient temperature to remove hydroxylamine and cofactor. The resulting apoenzyme was stored in 0.1 M potassium phosphate, 1 mM AET, 0.1 mM dithiothreitol, 10% (w/v) glycerol, pH 7.4 (buffer A) at 4°C. All experiments with apoenzyme were performed within 3 days after preparation.

RESULTS

Apoglutamate Decarboxylase Preparation

Apoglutamate decarboxylase can be prepared by hydroxylamine treatment of holoenzyme at low ionic strength. The resolution achieved is greater than 90%, and the yield of enzyme activity upon addition of pyridoxal phosphate is 90–100% (Table I). Addition of pyridoxal or pyridoxine fails to increase apoenzyme activity.

Molecular Weights

Apoenzyme, native holoenzyme, and reconstituted holoenzyme coeluted as a single peak of activity from a Sephadex G-200 column. The estimated molecular weight for all three enzyme preparations is 84,000 ± 2000 daltons (Figure 1).

Inhibition of Enzyme Activity by Sodium Borohydride and Salicylaldehyde

Pyridoxal phosphate is attached to the ε-amino group of a lysine residue as a Schiff base in a number of enzymes (16, 17). This group can be reduced with sodium borohydride, yielding a catalytically inactive product. Cerebellar hologlutamate decarboxylase, but not apoglutamate decarboxylase, is inactivated by sodium borohydride (Table II). At pH 7.4, holoenzyme is reduced by borohydride treatment to give a product which has 36% of the initial activity when assayed with excess cofactor. Under identical conditions, apoenzyme retains 72% of the original activity. At pH 6.3, the holoenzyme retains only 11%, whereas the apoenzyme retains 95% of the original activity.

Treatment of apoglutamate decarboxylase with salicylaldehyde inhibits enzyme activity. The apparent second-order rate constant for inactivation is 46.0 M⁻¹ min⁻¹ (data not shown). Holoenzyme, on the other hand, is resistant to inactivation by salicylaldehyde (Table III).
TABLE III
PROTECTION AGAINST SALICYLALDEHYDE INACTIVATION BY PYRIDOXAL PHOSPHATE

<table>
<thead>
<tr>
<th>Sample</th>
<th>Salicylaldehyde</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apoenzyme</td>
<td>−</td>
<td>10.2</td>
</tr>
<tr>
<td>Holoenzyme</td>
<td>−</td>
<td>10.2</td>
</tr>
<tr>
<td>Apoenzyme</td>
<td>+</td>
<td>3.6</td>
</tr>
<tr>
<td>Holoenzyme</td>
<td>+</td>
<td>8.6</td>
</tr>
</tbody>
</table>

*To 40 μl of apoglutamate decarboxylase solution in buffer A (90 μg protein, specific activity 10.9) was added 5 μl water (apoenzyme) or 10 mM pyridoxal phosphate (holoenzyme). After 1 min at 37°C, aliquots (5 μl) of 30 mM salicylaldehyde in 10% ethanol (v/v) or vehicle alone were added and the mixture was incubated 9 min more. The resulting solutions were assayed for specific activity as described in Experimental Procedure, using a final concentration of 0.5 mM pyridoxal phosphate.

Fig. 2. Reconstitution of cerebellar glutamate decarboxylase from apoenzyme pyridoxal phosphate. An aliquot (75 μl) of apoglutamate decarboxylase (5.0 mg/ml pr in buffer A, 91% resolved) was incubated 15 min at 37°C with 25 μl of 1 × 10^-4 M pyrazole phosphate. Assays were performed with 1 mM L-glutamate and a final concentration of 1.25 μM pyridoxal phosphate for the time specified (O). A similar experiment was cut out by incubating apoenzyme for 15 min without pyridoxal phosphate, then in cofactor (final concentration of 1.25 μM) just prior to assay (O).
Reconstitution of Holoenzyme Activity

Experiments with the mammalian enzyme demonstrate that formation of holoenzyme from apoenzyme and pyridoxal phosphate is rapid. Reconstitution of maximal activity is attained after incubating the apoenzyme 3–4 min in 1.25 μM pyridoxal phosphate (Figure 2). Similar results were obtained by lowering the glutamate concentration (in the assay) 25-fold, indicating that glutamate does not alter reconstitution. Using a 100-fold higher concentration of pyridoxal phosphate, reconstitution is too rapid to measure by this methodology.

DISCUSSION

The method for resolving cerebellar glutamate decarboxylase into apoenzyme and cofactor is quite unlike that used to resolve other pyridoxal phosphate enzymes (18, 19). The use of high phosphate or ammonium sulfate concentrations inhibits resolution with this enzyme where a low ionic strength is required.

Pyridoxal phosphate and its analogs cause reaggregation of rabbit muscle glycogen phosphorylase from apophosphorylase (20). However, pyridoxal phosphate does not play an apparent role in association or dissociation of glutamate decarboxylase subunits since apoenzyme and holoenzyme coelute from Sephadex G-200 columns. Mouse brain decarboxylase is composed of subunits (21), but information is lacking for the bovine brain enzyme.

Inactivation of holoenzyme, but not apoenzyme, by sodium borohydride is consistent with the hypothesis that the cofactor is bound in Schiff base linkage with the enzyme. Inactivation of apoenzyme, but not holoenzyme, by salicylaldehyde (a reagent which reacts reversibly with N-terminal α- and lysyl e-amino groups) supports this contention.

The binding of cofactor has been described for the bacterial decarboxylase. The enzyme from E. coli contains pyridoxal phosphate covalently linked to the e-amino group of a lysine residue at low pH as a Schiff base (22). The rate of cerebellar hologlutamate decarboxylase formation from apoenzyme and pyridoxal phosphate is much faster than that reported for the bacterial decarboxylase. The observed rate constant for E. coli holoenzyme formation is approximately 0.20 min⁻¹ at pH 4.9 (23). Complete reconstitution of E. coli decarboxylase requires about 2 hr using a high concentration (0.5 mM) of pyridoxal phosphate. The rate of holoenzyme formation for the cerebellar enzyme at pH 7.4 is too rapid
to measure by the enzyme assay technique, since the reaction is complete within a few minutes. Furthermore, spectral measurement of the reconstitution rate is unfeasible with the nonhomogeneous enzyme preparation. Assessment of the kinetics of reconstitution at pH 4.9 is unfeasible because the bovine brain enzyme is irreversibly inactivated at this pH.

These experiments provide direct evidence that pyridoxal phosphate is the cofactor for mammalian glutamate decarboxylase. Previous data in support of this contention were based in part on inhibition by carbonyl-trapping reagents. The resolution of the cerebellar decarboxylase and complete reconstitution of activity with pyridoxal phosphate reported here provides more definitive evidence that the enzyme requires pyridoxal phosphate as cofactor.

ACKNOWLEDGMENTS

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REFERENCES