Characterization of three kinetically distinct forms of glutamate decarboxylase from pig brain

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INTRODUCTION

Glutamate decarboxylase (l-glutamate 1-carboxylyase, EC 4.1.1.15) catalyses the synthesis of 4-aminobutyrate, a major inhibitory neurotransmitter in the central nervous system. The activity of glutamate decarboxylase appears to be highly regulated in mammalian brain, as evidenced by changes in the rates of 4-aminobutyrate synthesis in vivo in response to changes in neuronal activity (Gale & Casu, 1981; Chapman & Evans, 1983). One level of regulation of this enzyme is thought to involve a complex interaction with the enzyme and its cofactor, pyridoxal 5'-phosphate. The enzyme is only partially saturated with cofactor in vivo (Miller et al., 1977), and in synaptosomal preparations membrane depolarization causes an increase in cofactor saturation of the enzyme (Miller & Walters, 1979) and increased 4-aminobutyrate synthesis (Gold & Roth, 1979). Apoenzyme is produced when the enzyme is incubated with physiological concentrations of the substrate, glutamate (Miller et al., 1978), or the product, 4-aminobutyrate (Porter & Martin, 1984). ATP is an inhibitor of the enzyme (Turský, 1970), and micromolar concentrations of this nucleotide appear to enhance apoenzyme formation (Seligmann et al., 1978; Meeley & Martin, 1983).

A second level of regulation of 4-aminobutyrate synthesis is thought to involve multiple forms of the enzyme. A number of kinetic studies of crude or partially purified glutamate decarboxylase have been difficult to interpret, and have led to proposals that there are multiple forms of the enzyme in brain that differ kinetically, especially in their interactions with pyridoxal 5'-phosphate (Susz et al., 1966; Bayón et al., 1977; Meeley & Martin, 1983; Denner & Wu, 1985). Characterization of these forms has been thwarted by lack of methods to resolve them physically. Apparent high-Mr and low-Mr forms of the enzyme were separated by gel-filtration chromatography, but no kinetic differences were observed (Wu et al., 1976).

Studies in this laboratory have demonstrated that three distinct forms of pig brain glutamate decarboxylase can be resolved by hydrophobic-interaction chromatography on phenyl-Sepharose and by preparative isoelectric focusing (Spink et al., 1983). These forms did not appear to be produced at any stage of the purification, and were also resolved when hypo-osmotic lysates of a synaptosomal fraction were chromatographed on phenyl-Sepharose. Since it was not apparent how they might be related to one another, the forms of the enzyme with pI values of 5.3, 5.5 and 5.8 were referred to simply as the α-, β- and γ-forms respectively. Similar multiple forms of the enzyme have also been resolved from extracts of human and rat brain (Spink & Martin, 1983). To investigate their possible functional significance, we have purified the three forms of the enzyme from pig brain and conducted a detailed comparison of their kinetic properties.

EXPERIMENTAL

Materials

DL-[1-14C]Glutamic acid was obtained from New England Nuclear and from Research Products International. DEAE-Sepacel, phenyl-Sepharose, heparin-Sepharose, QAE- (quaternary aminomethyl-)Sephadex and CNBr-activated Sepharose were purchased from Pharmacia Fine Chemicals. Acrylamide, NN'-methylene-bisacrylamide and hydroxyapatite (Bio-Gel HT) were from Bio-Rad Laboratories. L-Glutamic acid, pyridoxal 5'-phosphate, imidazole and o-dianisidine were purchased from Sigma Chemical Co. All other chemicals were reagent grade. Rabbit anti-(sheep IgG) serum and

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Purification of glutamate decarboxylase

The initial stages of the purification, including preparation of the crude extract and chromatography on DEAE-Sephacel and hydroxypatite, were performed essentially as described by Blindermann et al. (1978). All chromatography buffers contained 1 M-2-aminoethylisothiouronium bromide and 20 μM-pyridoxal 5'-phosphate. The peak of glutamate decarboxylase activity from hydroxypatite chromatography was dialysed against 50 mM-potassium phosphate buffer, pH 7.0, and then chromatographed on a 2.6 cm x 10 cm column of QAE-Sephadex. The enzyme was eluted with a 500 ml linear gradient of 50–275 mM-potassium phosphate buffer. The peak fractions of enzyme activity were pooled and loaded on a 2.6 cm x 10 cm column of phenyl-Sepharose, and the three forms of glutamate decarboxylase were separated by eluting the column with a decreasing phosphate buffer gradient (225 mM to 10 mM) and a simultaneously increasing glycerol gradient (0 to 50%, v/v) over 600 ml. Each of the three peaks of enzyme activity was individually pooled, dialysed against 5 mM-potassium phosphate buffer, pH 7.0, and chromatographed on a 0.9 cm x 15 cm column of heparin-Sepharose. This column was eluted with a linear gradient of 5–225 mM-potassium phosphate buffer. The peak of enzyme activity was pooled, concentrated by ultrafiltration with an Amicon PM-10 membrane, and stored at −20 °C in the presence of 40% (w/v) sucrose for later use in kinetic experiments. Protein concentration was determined by using the COOMassie Blue dye-binding method (Bradford, 1976). Glutamate decarboxylase activity was measured by the 14CO2-trapping technique (Albers & Brady, 1959) as previously described (Miller et al., 1978).

Electrophoresis

Non-denaturing polyacrylamide-gel electrophoresis was performed as described by Williams & Reisfeld (1964) with 7% polyacrylamide slabs gels. Proteins were stained with 0.2% Coomassie Blue. When the gels were to be assayed for glutamate decarboxylase activity 20 μM-pyridoxal 5'-phosphate and 1 mM reduced glutathione were added to the upper-electrode buffer. The stacking buffer system of Williams & Reisfeld (1964) was also used for preparative electrophoresis in 2.5% agarose gels. Proteins were located in these gels by fluorescence staining with 0.03% 8-anilinonaphthalene-1-sulfonic acid (Hartman & Udenfriend, 1969). The method of Laemmli (1970) was used for sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Proteins were located by silver staining (Merril et al., 1981). In some cases enzyme samples were treated with dimethyl suberimidate (5 mg/ml in 0.4 M-triethanolamine buffer, pH 8.0) for 3 h at 20 °C by the method of Davies & Stark (1970) before sodium dodecyl sulphate/polyacrylamide-gel electrophoresis.

Immunological techniques

Antibodies to pig brain glutamate decarboxylase were raised in a young ram. The enzyme was purified from pig brain up to the QAE-Sephadex step, followed by non-denaturing polyacrylamide-gel electrophoresis in 6% gels. Proteins were located by staining with 0.03% 8-anilinonaphthalene-1-sulfonic acid, and bands corresponding to enzyme activity were cut from the gels and homogenized in 0.15 M-NaCl. Gel fragments containing approx. 100 μg of enzyme were emulsified in Freund’s complete adjuvant and injected subcutaneously on days 1 and 2. Anti-(glutamate decarboxylase) antibody in the dialysed serum was assayed by immunoprecipitation (Christenson et al., 1972), with rabbit anti-(sheep IgG) antibody as the second antibody. Anti-(glutamate decarboxylase) antibody was detected in serum collected 15 days after immunization, and antiserum collected on day 19 was used in subsequent studies.

The specific antibodies to pig brain glutamate decarboxylase were purified from the antiserum by affinity chromatography on a column of glutamate decarboxylase linked to Sepharose 4B. To prepare the affinity column, the peak-specific-activity enzyme from chromatography of the γ-form of the enzyme on heparin-Sepharose was allowed to react with CNBr-activated Sepharose 4B at a concentration of 0.1 mg of protein/ml of packed Sepharose. The antiserum (5 ml) was pre-adsorbed on an acetone-dried powder of rat liver, and the IgG fraction obtained after (NH4)2SO4 precipitation at 33%, saturation was resuspended, dialysed against phosphate-buffered saline (0.15 M-NaCl/0.02 M-sodium phosphate buffer, pH 7.4) and applied to the affinity column (0.9 cm x 3 cm) at a flow rate of 0.5 ml/h. The column was then washed with 100 ml of phosphate-buffered saline followed by elution of the bound antibody with 0.1 M-glycine/HCl buffer, pH 2.5, containing 1 M-NaCl.

The affinity-purified antibody was used for immunoblotting of polyacrylamide gels by the method of Towbin et al. (1979). The antibody recovered from a single run of the affinity column (about 0.2 mg) was diluted to 75 ml in 10 mM-Tris/HCl buffer, pH 7.4, containing 0.15 M-NaCl, 5% (w/v) bovine serum albumin and 10% (v/v) foetal-calf serum. This solution was used as the first antibody in the immunoblotting procedure. Peroxidase-conjugated rabbit anti-(sheep IgG) antibody at a 1:500 dilution was used as the second antibody.

Kinetics

The enzyme was prepared for kinetic experiments as previously described (Meeley & Martin, 1983). Enzyme reaction mixtures were buffered with 50 mM-imidazole/25 mM-acetic acid or 50 mM-Hepes. The buffers also contained 1 mM-2-aminoethylisothiouronium bromide and other components as indicated. Reaction-rate data were analysed by the computer program of Cleland (1967) for the determination of Kcat for glutamate and KI for 4-aminobutyrate and glutarate. Time courses of glutamate-promoted and 4-aminobutyrate-promoted inactivation of the enzyme were performed as in an earlier study (Meeley & Martin, 1983), and apparent first-order rate constants were calculated by linear-regression analysis of the natural logarithm of the enzyme activity against time. Statistical evaluations of these data were performed as described by Zar (1974). Kcat for pyridoxal 5'-phosphate and the rate constant for re-activation of the apoenzyme by pyridoxal 5'-phosphate were estimated by fitting an integrated equation (derived in the Results section) to data from experiments in which enzyme activity was measured as a function of the pyridoxal 5'-phosphate concentration. The data points in all kinetic experiments are means of triplicate measurements.
Table 1. Purification of the multiple forms of glutamate decarboxylase from 661 g of pig brain

One unit of enzyme activity catalyses the production of 1 \( \mu \text{mol of CO}_2/\text{min} \) from 0.5 mm-glutamate at 37 °C in 30 mm-sodium phosphate buffer, pH 7.0.

<table>
<thead>
<tr>
<th>Step</th>
<th>Protein (mg)</th>
<th>Activity (units)</th>
<th>(10^3 \times \text{Specific activity (units/mg)})</th>
<th>Yield (%)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>12,000 g supernatant</td>
<td>4100</td>
<td>2.40</td>
<td>0.588</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>DEAE-Sephacel</td>
<td>409</td>
<td>2.30</td>
<td>5.62</td>
<td>95.8</td>
<td>9.56</td>
</tr>
<tr>
<td>Hydroxyapatite</td>
<td>110</td>
<td>2.02</td>
<td>18.4</td>
<td>84.2</td>
<td>31.3</td>
</tr>
<tr>
<td>QAE-Sephadex</td>
<td>11.8</td>
<td>0.900</td>
<td>77.0</td>
<td>37.5</td>
<td>131</td>
</tr>
<tr>
<td><strong>α-Form</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenyl-Sepharose</td>
<td>0.105</td>
<td>0.0300</td>
<td>292</td>
<td>1.25</td>
<td>497</td>
</tr>
<tr>
<td>Heparin-Sepharose</td>
<td>0.041</td>
<td>0.0138</td>
<td>337</td>
<td>0.575</td>
<td>573</td>
</tr>
<tr>
<td><strong>β-Form</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenyl-Sepharose</td>
<td>0.840</td>
<td>0.244</td>
<td>290</td>
<td>10.2</td>
<td>493</td>
</tr>
<tr>
<td>Heparin-Sepharose (peak fractions)</td>
<td>0.302</td>
<td>0.117</td>
<td>378</td>
<td>4.87</td>
<td>643</td>
</tr>
<tr>
<td>Phenyl-Sepharose</td>
<td>0.021</td>
<td>0.0154</td>
<td>730</td>
<td>0.642</td>
<td>1240</td>
</tr>
<tr>
<td><strong>γ-Form</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenyl-Sepharose</td>
<td>1.17</td>
<td>0.194</td>
<td>166</td>
<td>8.08</td>
<td>282</td>
</tr>
<tr>
<td>Heparin-Sepharose (peak fractions)</td>
<td>0.161</td>
<td>0.0614</td>
<td>381</td>
<td>2.56</td>
<td>648</td>
</tr>
<tr>
<td>Phenyl-Sepharose</td>
<td>0.050</td>
<td>0.0348</td>
<td>700</td>
<td>1.45</td>
<td>1190</td>
</tr>
</tbody>
</table>

RESULTS

Purification of glutamate decarboxylase

A typical purification of glutamate decarboxylase from pig brain is summarized in Table 1. Substitution of ion-exchange chromatography on QAE-Sephadex as step 4, rather than gel filtration on Sephadex G-200 as used previously (Spink et al., 1983), resulted in a greater purification at this stage, but caused a partial resolution of the three forms, often resulting in a lower yield of the α-form. The final step in the purification, chromatography on heparin-Sepharose, gave significant purification of the β- and γ-forms, especially of the enzyme eluted in the later part of the phosphate gradient (Fig. 1). The α-form did not bind to heparin-Sepharose as tightly as did the other two enzyme forms, and was eluted with 4–5 column volumes of 5 mm-phosphate buffer. Although heparin-Sepharose chromatography did not give appreciable purification of the α-form, it did provide a means to separate the α- and β-forms when they were incompletely resolved on phenyl-Sepharose. The most highly purified preparations of the α-, β- and γ-forms of the enzyme had specific activities of 0.48, 1.2 and 2.3 \( \mu \text{mol/min per mg of protein} \) at saturating substrate and in the absence of inhibition by phosphate.

Enzyme preparations were subjected to non-denaturing polyacrylamide-gel electrophoresis. Gels stained with Coomassie Blue showed a high degree of purity of the β- and γ-forms after heparin-Sepharose chromatography, although several minor contaminating proteins were present. Non-denaturing polyacrylamide-gel electrophoresis of the β-form at several stages of purification is shown in Fig. 2. The mobilities relative to Bromophenol Blue determined by enzyme assay were 0.32, 0.29 and 0.25 for the α, β- and γ-forms respectively. Immunoblots of the non-denaturing gels showed a single band for each of the three enzyme forms that co-migrated with enzyme activity, as shown for the β-form (Fig. 2).

The β-form was further purified to apparent homo-
genity by agarose-gel electrophoresis. The enzyme recovered after this step migrated as a single band with an apparent Mr of 60000 on electrophoresis in sodium dodecyl sulphate/polyacrylamide gels (Fig. 3). Immuno-blots of sodium dodecyl sulphate/polyacrylamide gels after electrophoresis showed a single 60000-Mr band for each of the three enzyme forms and the 25000 g supernatant from pig brain (Fig. 3). After treatment with the cross-linking reagent dimethyl suberimidate, the β-form showed an additional band of Mr 120000 (Fig. 3e). Identical results were obtained for the α- and γ-forms after treatment with dimethyl suberimidate.

**Kinetics of glutamate decarboxylase**

Kinetic studies revealed highly significant differences among the properties of the three forms of the enzyme. The pH-dependence of the apparent Km for L-glutamate was determined in a constant-ionic-strength buffer prepared by titrating 25 mM-acetic acid with imidazole to the desired pH. Throughout the imidazole buffering range the apparent Km for glutamate was lowest for the α-form and highest for the γ-form (Fig. 4). The apparent Km for glutamate of each form showed a steep rise at pH 6.1, and the lowest value of Km for each form was obtained at pH 7.6. The effects of pH on Vmax of the three enzyme forms were quite similar; in each case the highest value of Vmax occurred at pH 6.2–6.5 (Fig. 4 inset).

The steady-state kinetic properties of glutamate decarboxylase were also investigated with Heps buffer.

The Km values for glutamate of the three enzyme forms showed the same trend in Hepes buffer as was observed in the imidazole/acetic acid buffers: the Km of the α-form was the lowest, and the Km of the γ-form was the highest (Table 2). The values of Km obtained in Hepes buffer were lower than those observed in imidazole/acetic acid buffers owing to competitive inhibition of the enzyme by acetate. 4-Aminobutyrate, the reaction product, was competitive with glutamate for each enzyme form. The Kl values for 4-aminobutyrate, glutarate and acetate showed the same trend among the three forms of the enzyme as did Km for L-glutamate, with the α-form having the lowest Kl and the γ-form having the highest Kl (Table 2).

Pyridoxal 5'-phosphate was found to be tightly bound to the three forms of glutamate decarboxylase, and could not be resolved from any of them by dialysis. Experiments with continuous-flow dialysis against buffer without pyridoxal 5'-phosphate at 30 °C resulted in loss of enzyme activity of the α-, β- and γ-forms with a rate constant less than 0.001 min⁻¹. Greater than 95% recovery of holoenzyme was obtained with each enzyme form after overnight dialysis at 4 °C against buffer without pyridoxal 5'-phosphate.

Addition of glutamate to dialysed enzyme preparations caused a time-dependent loss of activity of each form of the enzyme. Analysis of this glutamate-promoted inactivation revealed additional kinetic differences among the three forms. With enzyme preparations...
Kinetically distinct forms of brain glutamate decarboxylase

Fig. 3. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and immunoblotting of glutamate decarboxylase

The β-form after agarose-gel electrophoresis was applied to a 10% polyacrylamide gel (0.75 mm thick), electrophoresed in the presence of 0.1% sodium dodecyl sulphate, and the gel was stained with silver (a). (b)–(g) Immunoblotts of glutamate decarboxylase transferred from 10% polyacrylamide resolving gels. Samples were: (b) the 25000 g supernatant from pig brain; (c) the α-form from phenyl-Sepharose; (d) the β-form from phenyl-Sepharose; (e) the β-form from phenyl-Sepharose after treatment with dimethyl suberimidate (5 mg/ml); (f) and (g) an immunoblot of a 7.5% polyacrylamide resolving gel of the β-form (f) and γ-form (g) from phenyl-Sepharose. Arrows indicate the migration of Mr standards (soya-bean trypsin inhibitor, 20100; carbonic anhydrase, 30000; ovalbumin, 43000; bovine serum albumin, 67000; phosphorylase b, 94000; dimer of bovine serum albumin, 134000), which were run in adjacent lanes.

Fig. 4. Effect of pH on the $K_m$ and $V_{max}$. of glutamate decarboxylase

Samples of the α-, β- and γ-forms were exhaustively dialysed against imidazole/acetate buffers at the indicated pH values and assayed with various concentrations of glutamate from 0.5 to 10 mM (specific radioactivity 0.11 Ci/mol). Apparent $K_m$ values were estimated by using the computer program of Cleland (1967); standard errors are shown when larger than the symbol. The inset shows the relative $V_{max}$ plotted as a function of pH. □, α-form; ○, β-form; Δ, γ-form.
Table 2. Kinetic constants of brain glutamate decarboxylase

Values are the means ± S.E.M.

<table>
<thead>
<tr>
<th>Kinetic constant</th>
<th>α-Form</th>
<th>β-Form</th>
<th>γ-Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_m$, L-glutamate (mm)*</td>
<td>0.174 ± 0.015</td>
<td>0.451 ± 0.046§</td>
<td>1.237 ± 0.224†</td>
</tr>
<tr>
<td>$K_i$, 4-aminobutyrate (mm)*</td>
<td>11.1 ± 1.6</td>
<td>19.8 ± 4.1</td>
<td>23.9 ± 2.9†</td>
</tr>
<tr>
<td>$K_i$, glutarate (mm)*</td>
<td>1.29 ± 0.10</td>
<td>2.12 ± 0.39</td>
<td>3.72 ± 0.36†</td>
</tr>
<tr>
<td>$K_i$, acetate (mm)*</td>
<td>14.1 ± 4.6</td>
<td>18.4 ± 5.0</td>
<td>19.4 ± 3.5</td>
</tr>
<tr>
<td>$k_i$, L-glutamate (min⁻¹)†</td>
<td>0.0190 ± 0.0007</td>
<td>0.0424 ± 0.0017§</td>
<td>0.0907 ± 0.0037†</td>
</tr>
<tr>
<td>$k_i$, 4-aminobutyrate (min⁻¹)†</td>
<td>0.0202 ± 0.0017</td>
<td>0.0402 ± 0.0012§</td>
<td>0.0882 ± 0.0037†</td>
</tr>
<tr>
<td>$k_r$, pyridoxal 5'-phosphate (min⁻¹)†</td>
<td>0.041 ± 0.08</td>
<td>0.214 ± 0.049§</td>
<td>0.469 ± 0.040†</td>
</tr>
<tr>
<td>$K_o$, pyridoxal 5'-phosphate (µM)†</td>
<td>0.180 ± 0.060</td>
<td>0.348 ± 0.041§</td>
<td>0.76 ± 0.18‡</td>
</tr>
</tbody>
</table>

* Determined at 37 °C in 50 mM-Hepes buffer, pH 7.2.
† Determined at 30 °C in 50 mM-imidazole/25 mM-acetate buffer, pH 7.2.
‡ Significantly different from the value for the α-form ($P < 0.01$) by the two-tailed Student’s $t$ test.
§ Significantly different from the value for the γ-form ($P < 0.01$).
|| Significantly different from the value for the γ-form ($P < 0.05$).

Fig. 5. Inactivation of glutamate decarboxylase by saturating glutamate and 4-aminobutyrate

Enzyme samples in imidazole/acetate buffer, pH 7.2, were incubated at 30 °C in the presence of 10 mM-glutamate in (a), or 200 mM-4-aminobutyrate in (b), for various periods of time. Residual activity was then determined by a 5 min assay on addition of 0.11 µCi of [1-¹⁴C]glutamate. □, α-Form; O, β-form; △, γ-form.

present in the active form ($E_a/E_T$) as a function of time and the concentration of pyridoxal 5'-phosphate (eqn. 2, below) to experimental values of $E_a/E_T$ measured over a wide range of pyridoxal 5'-phosphate concentrations at various times of incubation. This equation was based on the following considerations. Since pyridoxal 5'-phosphate is tightly bound to the enzyme, simple association and dissociation of the cofactor is negligible and the major pathway of interaction of pyridoxal 5'-phosphate with the enzyme can be described as a cyclic process in which apoenzyme is formed by the alternative transamination reaction described in the accompanying paper (Porter et al., 1985) and holoenzyme is formed by reaction of the apoenzyme with pyridoxal 5'-phosphate.

The cycle can be represented as:

\[
\begin{align*}
E_a & \xrightarrow{k_r} (E - PLP) \\
(E - PLP) & \xrightarrow{+ \text{PLP}} E \\
E & \xrightarrow{k_i} E_{a/\text{PLP}} \\
E_{a/\text{PLP}} & \xrightarrow{k_i} E_a
\end{align*}
\]

In this mechanism $E_a$ is the active form of the enzyme
and E the apoenzyme. Activation takes place by a two-step process involving a rapid, reversible, equilibrium binding of pyridoxal 5'-phosphate (indicated by PLP) with the apoenzyme followed by a slow conversion of the (E–PLP) complex into active holoenzyme. The dissociation constant for the (E–PLP) complex is given by \( K_a \), the rate constant for activation of the (E–PLP) complex by \( k_r \) and the apparent first-order rate constant for inactivation by \( k_i \). Although the rate of inactivation actually depends on the glutamate concentration (Meeley & Martin, 1983), the experiments were carried out with fixed, saturating, concentrations of glutamate, and so terms for glutamate were included and the apparent rate constant was used. At saturating glutamate the rate of inactivation is equal to \( -k_i \, E_a \) and the rate of re-activation is equal to \( k_r \, (E–PLP) \). Since total inactive enzyme, \( E_i \), is equal to \( E + (E–PLP) \):

\[
\frac{dE_a}{dt} = -k_i \, E_a + \frac{k_r \, E_a \, (PLP)}{K_d + (PLP)}
\]

(1)

Total enzyme, \( E_T \), is equal to \( E_a + E_i \). After substitution to eliminate \( E_i \), the differential equation was integrated over time, yielding the final equation:

\[
\frac{E_{at}}{E_T} = \frac{E_{ao} \, e^{-k_i \, t}}{E_T} + \frac{k_r \, (PLP) \, (1 - e^{-k_i \, t})}{k'[K_d + (PLP)]}
\]

(2)

where

\[
k' = k_i + \frac{k_r \, (PLP)}{K_d + (PLP)}
\]

(3)

According to this model the limiting (steady-state) value of \( E_a/E_T \) at saturating pyridoxal 5'-phosphate is \( k_r/(k_i + k_r) \).

To investigate whether a mechanism without a saturable step could satisfactorily describe the data, the corresponding equation for a non-saturable mechanism of re-activation was derived and fitted to the data. This re-activation mechanism was a simple bimolecular reaction:

\[
E + PLP \xrightarrow{k_r} E_a
\]

(4)

in which the active form of the enzyme is formed directly by reaction of pyridoxal 5'-phosphate and apoenzyme. According to this model the limiting value of \( E_a/E_T \) at high concentrations of pyridoxal 5'-phosphate is 1.

The integrated equations were fitted to data and the kinetic constants estimated by a least-squares method by using a computer program based on the Levenburg–Marquardt algorithm. Experiments were carried out over a wide range of pyridoxal 5'-phosphate concentrations and with times of incubation ranging from 20 to 130 min (Fig. 6). In all cases the first model (eqn. 2) provided a good fit to the data. At low pyridoxal 5'-phosphate concentrations (< 10 nm) there was little re-activation by pyridoxal 5'-phosphate and the amount of active enzyme observed was in agreement with amounts predicted from the previously determined rates of glutamate-promoted inactivation. At higher concentrations of pyridoxal 5'-phosphate the amounts of active enzyme increased until a plateau was reached at which the observed \( E_a/E_T \) was significantly less than 1. The average (± S.D.) limiting values of \( E_a/E_T \) were 0.63 ± 0.08 (n = 5), 0.83 ± 0.09 (n = 4) and 0.86 ± 0.08 (n = 6) for the \( \alpha \)-, \( \beta \)- and \( \gamma \)-forms of the enzyme at saturating pyridoxal 5'-phosphate. The equations for the non-saturable re-activation (second) model fit the data at low concentrations of pyridoxal 5'-phosphate, where re-activation was insignificant. However, the fit was very poor at high concentrations of pyridoxal 5'-phosphate, since the observed values of \( E_a/E_T \) were significantly lower than the value 1 predicted by the integrated equation for this model.

A summary of the kinetic constants obtained for all three forms is presented in Table 2. The qualitative pattern in the variation among the forms is the same for every kinetic parameter; the kinetic parameters were in the order \( \gamma \)-form > \( \beta \)-form > \( \alpha \)-form of the enzyme. The greatest range is for \( k_r \), the first-order rate constant for activation of the apoenzyme by pyridoxal 5'-phosphate (about 11-fold) and the smallest range is for the inhibition constants for acetate (about 1.4-fold).

**DISCUSSION**

The results of this study indicate a subunit \( M_r \) of 60000 for each of the three forms of glutamate decarboxylase.
from pig brain. Cross-linking experiments with dimethyl suberimidate suggest a dimeric structure for the enzyme forms, which is consistent with estimations of the native enzyme \( M_r \) values for each form by gel-filtration chromatography (Spink et al., 1983). This dimeric subunit structure is similar to those reported for glutamate decarboxylases from human and rat brain (\( M_r \)

\[ 67000 \] for the monomers), which were based on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and gradient polyacrylamide-gel electrophoresis (Blindermann et al., 1978; Maitre et al., 1978). A dimeric subunit structure was initially proposed for the enzyme from mouse brain (Wu et al., 1973), although the monomer was substantially smaller (\( M_r \) 44000). A hexameric subunit structure (\( M_r \) 15000 for the monomer) was subsequently proposed on the basis of a complex pattern observed on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Matsuda et al., 1973). We obtained no evidence for such small monomers with the pig brain enzyme.

The kinetic properties of the three forms of pig brain glutamate decarboxylase are clearly different, indicating that the arrangement of important functional groups differs among the active sites of the three forms. The large variation among the forms of the rate of inactivation (about 5-fold) and the corresponding large variation of the ratio of decarboxylation to transamination (Porter et al., 1985) suggests that a group that protonates the quinoid intermediate could be in a slightly different position with respect to the intermediate in each form of the enzyme. However, the differences among the active sites appear unlikely to result from a simple shift in the orientation of a single catalytic group, since the \( K_i \) for glutarate, which does not undergo catalysis, shows significant differences among the forms. In a similar way the variations in the \( K_m \) for pyridoxal 5'-phosphate binding and the rate constant for reactivation of apoenzyme by pyridoxal 5'-phosphate are doubtless due to differences in the orientation of active-state functional groups among the three enzyme forms. Furthermore, the variations in the \( K_m \) for glutamate and the \( K_i \) values for acetate, 4-aminobutyrate, and glutarate which show the same trend among the forms (\( \alpha \)-form < \( \beta \)-form < \( \gamma \)-form) but differ considerably in magnitude, appear to be related to the complexity of the interactions of these compounds with the active site. The \( K_i \) for acetate, which probably reflects binding at one or both of the carboxy-group-binding sites in the active site, varies the least among the forms (1.4-fold). The \( K_m \) for glutamate, which may reflect binding of two carboxy groups and transaldimination of the enzyme-bound pyridoxal 5'-phosphate by the amino group, varies the most (7-fold). The variation in the \( K_i \) values for glutarate and 4-aminobutyrate, which have two functional groups to interact, is intermediate (2-3-fold).

The rates of inactivation by glutamate and 4-aminobutyrate differed among the three forms, yet the kinetics of inactivation of each form by saturating glutamate and 4-aminobutyrate were identical. These results suggest that enzyme inactivation promoted by the two compounds involves a common reaction intermediate at the active site. The fact that saturating 4-aminobutyrate does not inactivate any of the enzyme forms more readily than does saturating glutamate implies the existence of a slow step occurring after decarboxylation in the catalytic mechanism. If product release were much more rapid than decarboxylation, saturating concentrations of 4-aminobutyrate might be expected to increase the concentration of post-decarboxylation reaction intermediate(s), and thus cause a more rapid enzyme inactivation than saturating concentrations of glutamate. At present the most plausible mechanism for the inactivation of glutamate decarboxylase by glutamate is that of decarboxylation-dependent transamination, which has been proposed for other amino acid decarboxylases. In the accompanying paper (Porter et al., 1985) we present evidence that the multiple forms of glutamate decarboxylase catalyse the decarboxylation-dependent transamination of glutamate and the direct transamination of 4-aminobutyrate.

The large differences in the kinetic properties among the three enzyme forms suggest that they could catalyse differing rates of 4-aminobutyrate synthesis in vivo, dependent on such factors as the concentrations of substrate, product and cofactor. At present it is not known whether the three forms are products of distinct genes, or whether they might be interconverted by post-translational processing. It is possible that these forms represent another level of regulation of this enzyme. The further characterization of these enzyme forms may help to elucidate the cellular mechanisms controlling 4-aminobutyrate synthesis in the central nervous system.

REFERENCES

Kinetically distinct forms of brain glutamate decarboxylase


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