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

David C. SPINK, Terence G. PORTER, Susan J. WU and David L. MARTIN*

Wadsworth Center for Laboratories and Research, New York State Department of Health, Albany, NY 12201, U.S.A.

Pig brain contains three forms of glutamate decarboxylase with pI values of 5.3, 5.5 and 5.8, referred to as the α -, β - and γ -forms respectively. These forms were purified and kinetically characterized. The major synaptic form of glutamate decarboxylase (the β -form) migrated as a single band on electrophoresis in sodium dodecyl sulphate/polyacrylamide gels with an apparent M_r of 60000. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis followed by immunoblotting with an affinity-purified antibody to the enzyme indicated a subunit M_r of 60000 for the α - and γ -forms as well. An extensive kinetic analysis, aided by an integrated equation that describes the inactivation and re-activation cycle of the enzyme, revealed that the three forms of the enzyme differ markedly in kinetic properties. The K_m values for L-glutamate were 0.17, 0.45 and 1.24 mM respectively for the α -, β - and γ -forms. The K_i for 4-aminobutyrate, the first-order rate constants for inactivation by L-glutamate and 4-aminobutyrate, the rate constant for re-activation of the apoenzyme by pyridoxal 5'-phosphate and the dissociation constant for pyridoxal 5'-phosphate also differed in a similar way among the three forms; the values were in the order α -form < β -form < γ -form.

INTRODUCTION

Glutamate decarboxylase (L-glutamate 1-carboxylase, 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 of the enzyme with 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- M_r and low- M_r 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-Sepharose, phenyl-Sepharose, heparin-Sepharose, QAE- (quaternary aminoethyl-)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

* To whom correspondence should be addressed.

peroxidase-conjugated IgG fraction from rabbit anti-(sheep IgG) serum were obtained from Cappel Laboratories (Cooper Biomedical, Malvern, PA, U.S.A.).

Purification of glutamate decarboxylase

The initial stages of the purification, including preparation of the crude extract and chromatography on DEAE-Sephacel and hydroxyapatite, were performed essentially as described by Blindermann *et al.* (1978). All chromatography buffers contained 1 mM-2-aminoethylisothiuronium bromide and 20 μ M-pyridoxal 5'-phosphate. The peak of glutamate decarboxylase activity from hydroxyapatite chromatography was dialysed against 50 mM-potassium phosphate buffer, pH 7.0, and then chromatographed on a 2.6 cm $\times$ 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 $\times$ 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 $\times$ 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 the Coomassie Blue dye-binding method (Bradford, 1976). Glutamate decarboxylase activity was measured by the $^{14}\text{CO}_2$ -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 slab gels. Proteins were stained with 0.2% Coomassie Blue. When the gels were to be assayed for glutamate decarboxylase activity 20 μ M-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-anilino-naphthalene-1-sulphonic acid (Hartman & Udenfriend, 1969). The method of Laemmli (1970) was used for sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Proteins were located by silver staining (Merrill *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.

Immunochemical 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-anilino-naphthalene-1-sulphonic acid, and bands corresponding to enzyme activity were cut from the gels and homogenized in 0.15 M-NaCl. Gel fragments containing approx. 100 μ g of enzyme were emulsified in Freund's complete adjuvant and injected subcutaneously on days 1 and 12. 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 from rat liver, and the IgG fraction obtained after $(\text{NH}_4)_2\text{SO}_4$ 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 $\times$ 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). Enzymic reaction mixtures were buffered with 50 mM-imidazole/25 mM-acetic acid or 50 mM-Hepes. The buffers also contained 1 mM-2-aminoethylisothiuronium bromide and other components as indicated. Reaction-rate data were analysed by the computer program of Cleland (1967) for the determination of K_m for glutamate and K_i 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). K_d 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 μmol of CO_2/min from 0.5 mM-glutamate at 37 °C in 30 mM-sodium phosphate buffer, pH 7.0.

Step	Protein (mg)	Activity (units)	$10^3 \times$ Specific activity (units/mg)	Yield (%)	Purification (fold)
12000 g supernatant	4100	2.40	0.588	100	1
DEAE-Sephacel	409	2.30	5.62	95.8	9.56
Hydroxyapatite	110	2.02	18.4	84.2	31.3
QAE-Sephadex	11.8	0.900	77.0	37.5	131
α -Form					
Phenyl-Sephadex	0.105	0.0300	292	1.25	497
Heparin-Sephadex	0.041	0.0138	337	0.575	573
β -Form					
Phenyl-Sephadex	0.840	0.244	290	10.2	493
Heparin-Sephadex	0.302	0.117	378	4.87	643
(peak fractions)	0.021	0.0154	730	0.642	1240
γ -Form					
Phenyl-Sephadex	1.17	0.194	166	8.08	282
Heparin-Sephadex	0.161	0.0614	381	2.56	648
(peak fractions)	0.050	0.0348	700	1.45	1190

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-Sephadex, 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-Sephadex as tightly as did the other two enzyme forms, and was eluted with 4–5 column volumes of 5 mM-phosphate buffer. Although heparin-Sephadex 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-Sephadex. 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 $\mu\text{mol}/\text{min}$ 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-Sephadex 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-

Fig. 1. Heparin-Sephadex chromatography of glutamate decarboxylase

(a) The α fraction from phenyl-Sephadex was dialysed against 5 mM-potassium phosphate buffer, pH 7.0, and applied to a 0.5 cm $\times$ 15 cm column of heparin-Sephadex. The α -form was eluted with 50 ml of 5 mM-potassium phosphate buffer, followed by a linear gradient up to 150 mM-potassium phosphate, which eluted the small amount of the β -form of the enzyme contaminating the α peak from phenyl-Sephadex. (b) Chromatography of the γ -form from phenyl-Sephadex on heparin-Sephadex. ●, Enzyme activity; ○, protein; —, phosphate concentration.

Fig. 2. Polyacrylamide-gel electrophoresis of glutamate decarboxylase under non-denaturing conditions

(a) Coomassie Blue-stained gel containing glutamate decarboxylase at the hydroxyapatite stage of purification (lane 1), the β peak from phenyl-Sepharose (lane 2) and the β -form after heparin-Sepharose chromatography (lane 3). (b) A lane containing the heparin-Sepharose-purified β -form was cut into 3 mm slices, each of which was placed in 100 μ l of 100 mM-sodium phosphate buffer, pH 7.0, and assayed for glutamate decarboxylase activity with 0.5 mM-glutamate and 0.11 μ Ci of [1- 14 C]glutamate. (c) The β -form at the phenyl-Sepharose stage of purification was transferred from the non-denaturing gel on to nitrocellulose and immunochemically detected by using the affinity-purified anti-(glutamate decarboxylase) antibody (3 μ g/ml) and peroxidase-conjugated rabbit anti-(sheep IgG) serum at a 1:500 dilution.

geneity by agarose-gel electrophoresis. The enzyme recovered after this step migrated as a single band with an apparent M_r of 60000 on electrophoresis in sodium dodecyl sulphate/polyacrylamide gels (Fig. 3). Immunoblots of sodium dodecyl sulphate/polyacrylamide gels after electrophoresis showed a single 60000- M_r 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 M_r 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 K_m 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 K_m for glutamate was lowest for the α -form and highest for the γ -form (Fig. 4). The apparent K_m for glutamate of each form showed a steep rise at pH 6.1, and the lowest value of K_m for each form was obtained at pH 7.6. The effects of pH on V_{max} of the three enzyme forms were quite similar; in each case the highest value of V_{max} occurred at pH 6.2–6.5 (Fig. 4 inset).

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

The K_m 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 K_m of the α -form was the lowest, and the K_m of the γ -form was the highest (Table 2). The values of K_m 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 K_i values for 4-aminobutyrate, glutamate and acetate showed the same trend among the three forms of the enzyme as did K_m for L-glutamate, with the α -form having the lowest K_i and the γ -form having the highest K_i (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

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) Immunoblots 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 suberimide (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 M_r 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

containing less than 1 μ M added pyridoxal 5'-phosphate, glutamate caused a first-order loss of enzyme activity (Fig. 5a). The γ -form was inactivated 2-fold faster than the β -form and more than 4-fold faster than the α -form. The pseudo-first-order rate constants for inactivation by 10 mM-glutamate are presented in Table 2. These differences in rates cannot be explained by differences in the degrees of saturation with substrate, since each form was more than 85% saturated with glutamate. Furthermore, the α -form has the lowest K_m for glutamate and would therefore be the most highly saturated, yet this form showed the lowest rate of glutamate-promoted inactivation. Conversely the γ -form was the most rapidly inactivated despite a 7-fold higher K_m for substrate than that of the α -form. Glutamate decarboxylase is also inactivated by saturating concentrations of 4-aminobutyrate (Porter & Martin, 1984). When pyridoxal 5'-phosphate was not present in the incubation medium each of the enzyme forms was inactivated by a first-order process, but again at substantially different rates. The observed kinetics of 4-aminobutyrate-promoted inactivation were indistinguishable from those of glutamate-promoted inactivation (Fig. 5b).

The kinetic constants for the activation of apoenzyme by pyridoxal 5'-phosphate were estimated by fitting an integrated equation that gives the fraction of enzyme

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. $\square$, α -Form; $\circ$, β -form; $\triangle$, γ -form.

Table 2. Kinetic constants of brain glutamate decarboxylaseValues are the means $\pm$ S.E.M.

Kinetic constant	α -Form	β -Form	γ -Form
K_m , L-glutamate (mM)*	0.174 ± 0.015	$0.451 \pm 0.046^{\dagger}\S$	$1.237 \pm 0.224^{\dagger}$
K_i , 4-aminobutyrate (mM)*	11.1 ± 1.6	19.8 ± 4.1	$23.9 \pm 2.9^{\dagger}$
K_i , glutarate (mM)*	1.29 ± 0.10	2.12 ± 0.39	$3.72 \pm 0.36^{\dagger}$
K_i , acetate (mM)*	14.1 ± 4.6	18.4 ± 5.0	19.4 ± 3.5
k_i , L-glutamate (min^{-1}) $\dagger$	0.0190 ± 0.0007	$0.0424 \pm 0.0017^{\dagger}\S$	$0.0907 \pm 0.0037^{\dagger}$
k_i , 4-aminobutyrate (min^{-1}) $\dagger$	0.0202 ± 0.0017	$0.0402 \pm 0.0012^{\dagger}\S$	$0.0882 \pm 0.0037^{\dagger}$
k_r , pyridoxal 5'-phosphate (min^{-1}) $\dagger$	0.041 ± 0.08	$0.214 \pm 0.049^{\parallel}$	$0.469 \pm 0.040^{\dagger}$
K_d , pyridoxal 5'-phosphate (μM) $\dagger$	0.180 ± 0.060	$0.348 \pm 0.041^{\S}$	$0.76 \pm 0.18^{\dagger}$

* Determined at 37 °C in 50 mM-Hepes buffer, pH 7.2.

 $\dagger$ Determined at 30 °C in 50 mM-imidazole/25 mM-acetate buffer, pH 7.2. $\dagger$ Significantly different from the value for the α -form ($P < 0.01$) by the two-tailed Student's t test. $\S$ Significantly different from the value for the γ -form ($P < 0.01$). $\parallel$ 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 [$1\text{-}^{14}\text{C}$]glutamate. $\square$, α -Form; $\circ$, β -form; $\triangle$, 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:

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_d , 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 no 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 $E + (E-PLP)$:

$$\frac{dE_a}{dt} = -k_i E_a + \frac{k_r E_i (PLP)}{K_d + (PLP)} \quad (1)$$

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:

$$\frac{E_{at}}{E_T} = \frac{E_{a(0)} e^{-k't}}{E_T} + \frac{k_r (PLP) (1 - e^{-k't})}{k' [K_d + (PLP)]} \quad (2)$$

where

$$k' = k_i + \frac{k_r (PLP)}{K_d + (PLP)} \quad (3)$$

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:

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 ($\pm$ 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 α -, β - and γ -forms of the enzyme at saturating pyridoxal 5'-phosphate. The

Fig. 6. Dependence of glutamate decarboxylase on pyridoxal 5'-phosphate

(a) Dialysed enzyme samples were incubated at 30 °C for 30 min in the presence of 10 mM-glutamate and the indicated concentrations of pyridoxal 5'-phosphate, after which 0.55 μ Ci of [14 C]glutamate was added and enzyme activity measured in a 5 min assay. (b) Enzyme samples were incubated and assayed as in (a), except that incubation times were approximately 3 times the half-time for glutamate-promoted inactivation, specifically 130, 54, and 21 min for the α -, β - and γ -form respectively. The curves show the least-squares fit of eqn. 2 (see the text) to the data. Less than 10% of added pyridoxal 5'-phosphate was consumed during reactions conducted at concentrations of cofactor above 10 nM. $\square$, α -Form; $\circ$, β -form; $\triangle$, γ -form.

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 γ -form $>$ β -form $>$ α -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 suberimide 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_d 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 (α -form < β -form < γ -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 rapidly 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.

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