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Transaminations catalysed by brain glutamate decarboxylase

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In addition to normal decarboxylation of glutamate to 4-aminobutyrate, glutamate decarboxylase from pig brain was shown to catalyse decarboxylation-dependent transamination of L-glutamate and direct transamination of 4-aminobutyrate with pyridoxal 5′-phosphate to yield succinic semialdehyde and pyridoxamine 5′-phosphate in a 1:1 stoichiometric ratio. Both reactions result in conversion of holoenzyme into apoenzyme. With glutamate as substrate the rates of transamination differed markedly among the three forms of the enzyme (0.008, 0.012 and 0.029% of the rate of 4-aminobutyrate production by the α-, β- and γ-forms at pH 7.2) and accounted for the differences among the forms in rates of inactivation by glutamate and 4-aminobutyrate. Rates of transamination were maximal at about pH 8 and varied in parallel with the rate constants for inactivation from pH 6.5 to 8.0. Rates of transamination of glutamate and 4-aminobutyrate were similar, suggesting that the decarboxylation step is not entirely rate-limiting in the normal mechanism. The transamination was reversible, and apoenzyme could be reconstituted to holoenzyme by reverse transamination with succinic semialdehyde and pyridoxamine 5′-phosphate. As a major route of apoenzyme formation, the transamination reaction appears to be physiologically significant and could account for the high proportion of apoenzyme in brain.

INTRODUCTION

The interaction of brain glutamate decarboxylase with its cofactor pyridoxal 5′-phosphate appears to be centrally important to the regulation of the enzyme, and consequently to the control of the synthesis of 4-aminobutyrate, an inhibitory neurotransmitter. Although a major fraction of glutamate decarboxylase is present *in vivo* as the apoenzyme, the mechanism of formation of apoenzyme has not been established. The apoenzyme does not appear to be readily formed by simple dissociation of the cofactor, since little is formed during dialysis (Spink *et al.*, 1985). Evidence for apoenzyme formation by decarboxylation-dependent transamination (also termed abortive transamination, since it leads to enzyme inactivation) has been obtained for some other decarboxylases (Sukhareva & Braunstein, 1971; Borri Voltattorni *et al.*, 1971; O'Leary & Baughn, 1977; O'Leary & Herreid, 1978). This transamination is thought to result from an alternative protonation of an intermediate formed after the decarboxylation step, resulting in the conversion of pyridoxal 5′-phosphate into pyridoxamine 5′-phosphate and the decarboxylated amino acid to an aldehyde or a ketone. Apoenzyme is then formed by dissociation of the pyridoxamine 5′-phosphate. Evidence for this transamination reaction has included spectral demonstrations of the conversions of pyridoxal 5′-phosphate into pyridoxamine 5′-phosphate and chemical identification of pyridoxamine 5′-phosphate and the expected aldehyde or ketone. Although the stoichiometric ratio of pyridoxamine 5′-phosphate to the expected aldehyde or ketone should be 1:1, this has never been demonstrated for any decarboxylase. Similarly, other characteristic enzymic reactions that are expected from the mechanism, including transamination of the amine product of normal decarboxylation and reconstitution of the holenzyme by reaction with the aldehyde and pyridoxamine 5′-phosphate, have not been demonstrated for glutamate decarboxylase or other amino acid α-decarboxylases.

Previous studies showing that brain glutamate decarboxylase is inactivated by glutamate and 4-aminobutyrate and that added pyridoxal 5′-phosphate is required to maintain enzyme activity in the presence of substrate strongly suggested that the enzyme carries out the abortive transamination reaction (Miller *et al.*, 1978; Meeley & Martin, 1983; Porter & Martin, 1984). Owing to the potential importance of the transamination reaction in the regulation of brain glutamate decarboxylase activity and to understanding the mechanisms of amino acid α-decarboxylases in general, we investigated whether the brain enzyme catalyses the reaction. The results provide strong evidence for the transamination mechanism and help to account for the differences in rates of inactivation by glutamate and 4-aminobutyrate among the multiple forms of the enzyme.

EXPERIMENTAL

Materials

Bicine, iproniazid phosphate, NAD^+, NADH, pyridoxamine 5′-phosphate, $Na_2S_2O_4$ and succinic semialdehyde were obtained from Sigma Chemical Co.; chelidonic acid, 3,5-diaminobenzoic acid dihydrochloride and h.p.l.c.-grade sodium acetate were from Aldrich Chemical Co.; DL-gabaculine was from Calbiochem–Behring; h.p.l.c.-grade methanol was from Fisher Scientific Co.; *o*-phthalaldehyde was from Pierce Chemical Co. The α-, β- and γ-forms of glutamate decarboxylase were purified by the procedures given in the preceding paper (Spink *et al.*, 1985). All other reagents were obtained from the previously quoted sources (Spink *et al.*, 1985) or were of the highest purity commercially available.

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All absorbance measurements and spectra were recorded on a Perkin–Elmer model 552 spectrophotometer, and fluorescence measurements and spectra were recorded on a Perkin–Elmer model MPF-44B fluorescence spectrophotometer equipped with a DCSU-2 differential corrected spectra unit. A Waters μBondapak C-18 column (3.9 mm × 30 cm) was used for h.p.l.c. The column eluent was monitored with the MPF-44B fluorimeter equipped with a flow cell. Peak height, area and retention time were determined by a Hewlett–Packard model 3390A programmable integrator.

Spectroscopy

Absorbance spectra were obtained by incubating glutamate decarboxylase with 20 mM-glutamate in 50 mM-Hepes/NaOH buffer, pH 7.0, containing 1 mM-sodium phosphate, 1 mM-2-aminoethylisothiouronium bromide and 10 μM-pyridoxal 5′-phosphate in a total volume of 1 ml. Difference spectra were recorded in the range 300–500 nm at approx. 30 min intervals against a reference that contained no glutamate. Between scans the enzyme solutions were incubated at 30 °C in the dark to avoid photodegradation of pyridoxal 5′-phosphate and pyridoxamine 5′-phosphate. All solutions were deoxygenated by bubbling with N_2 before incubation.

To obtain fluorescence spectra glutamate decarboxylase was incubated in the dark, at 30 °C, in deoxygenated 50 mM-Hepes/NaOH buffer, pH 7.0, containing 1 mM-sodium phosphate, 1 mM-2-aminoethylisothiouronium bromide, 5 μM-pyridoxal 5′-phosphate and 10 mM-sodium glutamate. Samples (100 μl) were removed at regular time intervals, diluted to 3 ml with 10 mM-Hepes buffer and scanned for fluorescence emission in the range 350–500 nm at an excitation wavelength of 330 nm. A small background fluorescence due to protein and reagents was subtracted by using the differential scanning accessory. In the stoichiometry experiments pyridoxamine 5′-phosphate was quantified by measuring fluorescence emission at 400 nm, at an excitation wavelength of 330 nm.

Analysis of pyridoxamine 5′-phosphate, succinic semialdehyde and 4-aminobutyrate by h.p.l.c.

Pyridoxamine 5′-phosphate was determined as the *o*-phthalaldehyde/ethanethiol derivative by reverse-phase h.p.l.c. by using a modification of a method developed for the analysis of amino acids (Hill *et al.*, 1979). A 100 μl portion of the pyridoxamine 5′-phosphate standard or glutamate decarboxylase reaction mixture was incubated with 100 μl of the *o*-phthalaldehyde/ethanethiol derivative-formation solution for 3 min at room temperature. A 100 μl sample was then applied to a C-18 column and eluted with 50 mM-sodium acetate/phosphate buffer, pH 6.0, containing 10% (v/v) methanol at a flow rate of 1.5 ml/min. The fluorescence of the eluate was monitored continuously with excitation and emission wavelengths of 336 nm and 395 nm respectively. The retention time of the pyridoxamine 5′-phosphate derivative was 14.2 min and peak area was directly proportional to the amount of pyridoxamine 5′-phosphate over the range 5–500 pmol.

Succinic semialdehyde standards or glutamate decarboxylase reaction mixtures were incubated with 0.15 M-3,5-diaminobenzoic acid at 60 °C for 2 h and further incubated overnight at room temperature to form the quinaldine derivative (Velluz *et al.*, 1948). Samples (100 μl) were injected on to a C-18 column and eluted at room temperature with 0.1 M-sodium acetate buffer, pH 3.5, containing 7.5% (v/v) methanol at a flow rate of 1 ml/min. The eluate was continuously monitored for fluorescence with excitation and emission wavelengths of 405 and 505 nm respectively. The retention time of the quinaldine derivative was 14 min and the peak area was directly proportional to the amount of succinic semialdehyde over the range 5–500 pmol. Stock solutions of succinic semialdehyde were standardized enzymically with succinic semialdehyde dehydrogenase by the fluorimetric determination of NADH (Pitts & Quick, 1965). Succinic semialdehyde dehydrogenase was purified from rat brain (Cash *et al.*, 1977) to a specific activity of 33 units/mg. 4-Aminobutyrate was determined as the *o*-phthalaldehyde/ethanethiol derivative by the h.p.l.c. method of Hill *et al.* (1979).

Stoichiometry, time course and frequency of transamination

The α-, β- and γ-forms of glutamate decarboxylase were dialysed overnight against N_2-saturated 10 mM-potassium phosphate buffer, pH 7.2, containing 1 mM-2-aminoethylisothiouronium bromide and 1 mM-EDTA and concentrated in Amicon CF50A ultrafiltration membrane cones. After the addition of pyridoxal 5′-phosphate (10 μM final concentration) the reaction tubes were sealed and alternately evacuated and flushed with ultra-high-purity N_2 (five cycles) that had been passed over heated copper filings to remove residual O_2. Reactions were initiated by injecting glutamate (20 mM final concentration), and anaerobic conditions were ensured by the simultaneous addition of $Na_2S_2O_4$ (200 μM final concentration). Reactions were stopped by freezing on solid CO_2. The samples were rapidly thawed, the protein was removed by ultrafiltration, and the filtrate was analysed for pyridoxamine 5′-phosphate, succinic semialdehyde and 4-aminobutyrate. CO_2 production was determined radiometrically (Spink *et al.*, 1985) in separate reaction tubes. Zero-time and non-enzyme controls were routinely incorporated into each experiment. 4-Aminobutyrate experiments were performed in a similar manner except that 4-aminobutyrate (200 mM final concentration) replaced glutamate in the reaction mixture. In the pH study, 75 mM-Hepes/NaOH buffer was used at pH 6.5–7.0 and 75 mM-Bicine/NaOH buffer from pH 7.0 to 9.0.

Inactivation and re-activation

The inactivation study was performed in 50 mM-Hepes/NaOH buffer, pH 7.2, by a two-step procedure (Meeley & Martin, 1983). First, glutamate decarboxylase was preincubated with 10 mM-glutamate in the presence or in the absence of succinic semialdehyde (500 μM final concentration) and/or pyridoxamine 5′-phosphate (50 μM final concentration). At the end of this inactivation period, DL-[^{14}C]glutamate was added and the remaining activity determined in a 5 min assay. Re-activation was measured by a similar procedure. After inactivation for 30 min in the presence of 10 mM-glutamate and 50 μM-pyridoxamine 5′-phosphate, succinic semialdehyde (500 μM final concentration) was added and re-activation allowed to proceed for various lengths of time. Enzyme activity was then measured in a 5 min assay.

RESULTS

Identification and stoichiometry of the transamination products

The appearance of pyridoxamine 5′-phosphate, an expected product of decarboxylation-dependent transamination, and the concomitant disappearance of pyridoxal 5′-phosphate was observed spectrophotometrically during incubation of the γ-form of glutamate decarboxylase with glutamate and free pyridoxal 5′-phosphate (Fig. 1*a*). Although the absorbance change was small, a time-dependent appearance of a maximum at 320 nm corresponding to the production of pyridoxamine 5′-phosphate and an appearance of a minimum at 395 nm corresponding to a loss of pyridoxal 5′-phosphate were clearly observed in the difference spectra. The enzyme was quite stable under the incubation conditions and was still 75% active after 5 h of incubation. The isosbestic point at 350 nm indicated an absence of spectrally distinct intermediates and was consistent with a direct conversion of pyridoxal 5′-phosphate into pyridoxamine 5′-phosphate during the reaction. Because of its strong fluorescent properties, the production of pyridoxamine 5′-phosphate was much more readily demonstrated by fluorescence spectroscopy; the time-dependent appearance of a fluorescent species with spectral properties identical with pyridoxamine 5′-

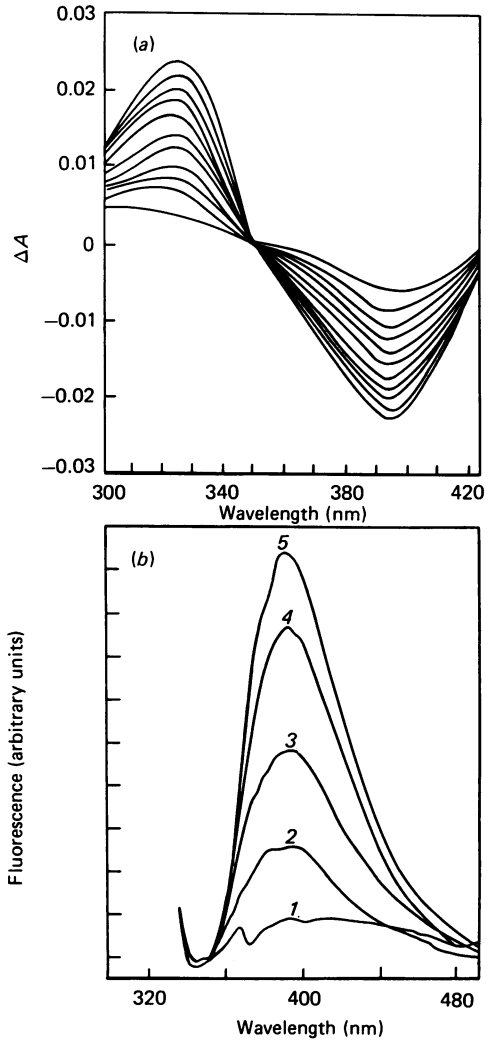


Fig. 1. Spectrophotometric demonstration of pyridoxamine 5′-phosphate production by glutamate decarboxylase

(*a*) Difference absorbance spectra recorded at 30 min intervals during the decarboxylation of 20 mM-glutamate by the γ-form of glutamate decarboxylase in the presence of 10 μM-pyridoxal 5′-phosphate, at 30 °C, in 50 mM-Hepes buffer containing 1 mM-sodium phosphate, 1 mM-EDTA and 1 mM-2-aminoethylisothiouronium bromide, pH 7.0. (*b*) Fluorescence spectra of a similar reaction mixture. Spectrum *1*, 0 min; spectrum *2*, 60 min; spectrum *3*, 110 min; spectrum *4*, 220 min. The fluorescence of 40 nM-pyridoxamine 5′-phosphate is also shown (spectrum *5*).

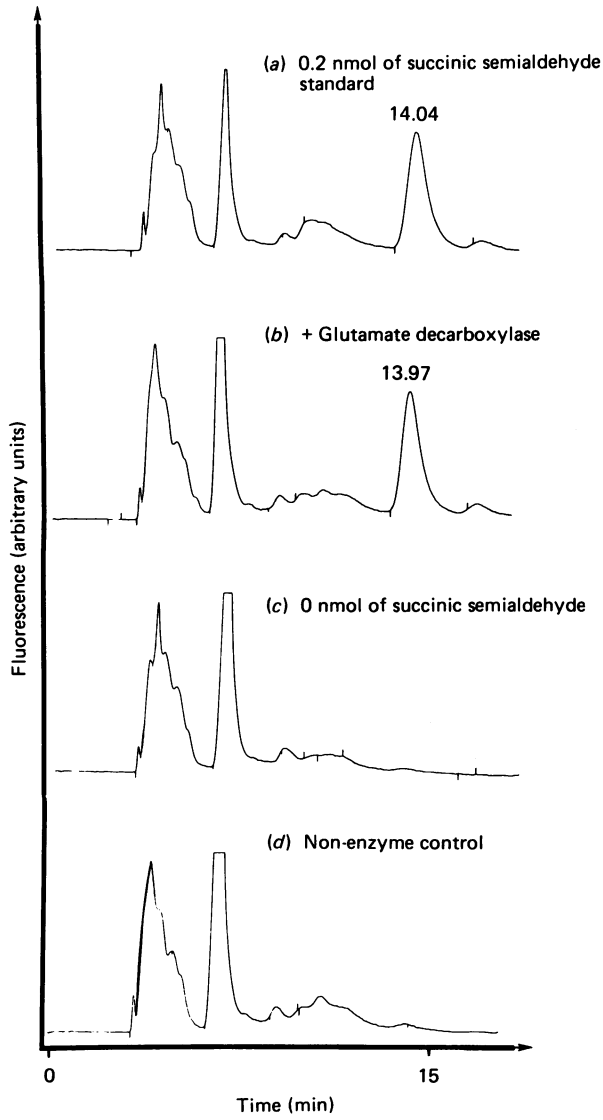


Fig. 2. Separation of the quinaldine derivative of succinic semialdehyde and 3,5-diaminobenzoic acid from the glutamate decarboxylase reaction mixture by reverse-phase h.p.l.c.

Details are described in the Experimental section. Chromatograms are of (*a*) 0.2 nmol of succinic semialdehyde standard, (*b*) glutamate decarboxylase reaction mixture after 180 min, (*c*) water blank and (*d*) reaction mixture without enzyme after 180 min.

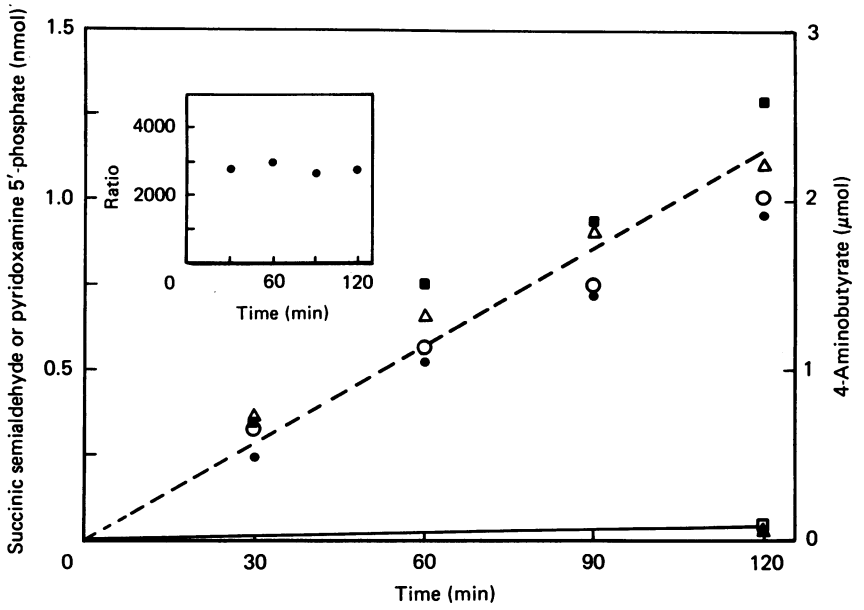


Fig. 3. Time course of pyridoxamine 5'-phosphate, succinic semialdehyde and 4-aminobutyrate production

The γ-form of glutamate decarboxylase was incubated with 20 mM-glutamate in the presence of 10 μM-pyridoxal 5'-phosphate at 30 °C in 10 mM-phosphate buffer, pH 7.8, containing 1 mM-2-aminoethylisothiouronium bromide, 1 mM-EDTA and 0.2 mM-$Na_2S_2O_4$. At the indicated times, samples were analysed for pyridoxamine 5'-phosphate (○), succinic semialdehyde (●) and 4-aminobutyrate (■) by the procedures described in the Experimental section. Pyridoxamine 5'-phosphate (▲) and succinic semialdehyde (□) production in non-enzyme controls are also shown. In addition, pyridoxamine 5'-phosphate production when 200 mM-4-aminobutyrate replaced glutamate in the reaction mixture is shown (△). The inset shows the ratio of 4-aminobutyrate to pyridoxamine 5'-phosphate production in the glutamate reaction mixture plotted as a function of time.

phosphate was observed when the enzyme was incubated with glutamate and pyridoxal 5'-phosphate (Fig. 1*b*). Further confirmation of pyridoxamine 5'-phosphate production was obtained by separating the *o*-phthalaldehyde/ethanethiol derivative of pyridoxamine 5'-phosphate from the reaction mixture by reverse-phase h.p.l.c. (results not shown). Quantification of pyridoxamine 5'-phosphate from the same reaction mixture by its native fluorescence and by the fluorescence of its *o*-phthalaldehyde/ethanethiol derivative agreed within 5%.

The production of succinic semialdehyde was demonstrated by reverse-phase h.p.l.c. of a similar enzyme reaction mixture (Fig. 2). After formation of the derivative with 3,5-diaminobenzoic acid a peak with a retention time identical with that of the standard succinic semialdehyde quinaldine derivative was observed (Figs. 2*a* and 2*b*). The early peaks in the chromatogram were due to components of the derivative-formation reagent as shown in the water blank (Fig. 2*c*), and there was negligible non-enzymic reaction under these conditions (Fig. 2*d*).

In control experiments, the production of pyridoxamine 5'-phosphate and succinic semialdehyde was shown to be absolutely dependent on the presence of the enzyme, L-glutamate and pyridoxal 5'-phosphate. Further, the production of pyridoxamine 5'-phosphate and succinic semialdehyde was completely inhibited by 1.0 mM-chelidonic acid, a potent competitive inhibitor of glutamate decarboxylase (Porter & Martin, 1985). Pretreatment of the enzyme with 50 μM-DL-gabaculine, an irreversible inhibitor of 4-aminobutyrate transaminase, or the addition of 1 mM-iproniazid, an inhibitor of monoamine oxidase, had no effect on the production of pyridoxamine 5'-phosphate or succinic semialdehyde. The production of succinic semialdehyde and pyridoxamine 5'-phosphate from reaction mixtures of 4-aminobutyrate, pyridoxal 5'-phosphate and glutamate decarboxylase was also demonstrated by using h.p.l.c.

The time course of pyridoxamine 5'-phosphate and succinic semialdehyde production during the reaction of glutamate decarboxylase with pyridoxal 5'-phosphate and glutamate remained linear over 2 h (Fig. 3), and the stoichiometric ratio of pyridoxamine 5'-phosphate and succinic semialdehyde was clearly 1:1. The ratio of 4-aminobutyrate to pyridoxamine 5'-phosphate formation remained constant at approx. 2700:1 during the incubation period (Fig. 3 inset), which is equivalent to a transamination frequency of 0.04% of decarboxylations. Under these conditions negligible non-enzymic reaction occurred. At the same concentration of enzyme, saturating concentrations of 4-aminobutyrate and glutamate produced similar amounts of pyridoxamine 5'-phosphate (Fig. 3) and a 1:1 ratio of succinic semialdehyde and pyridoxamine 5'-phosphate was observed when the enzyme was incubated with pyridoxal 5'-phosphate and 4-aminobutyrate.

Reversibility and pH-dependence of the transamination pathway

The rate of inactivation of the enzyme by glutamate was retarded about 3-fold by 500 μM-succinic semialdehyde (Fig. 4*a*). Pyridoxamine 5'-phosphate alone had no

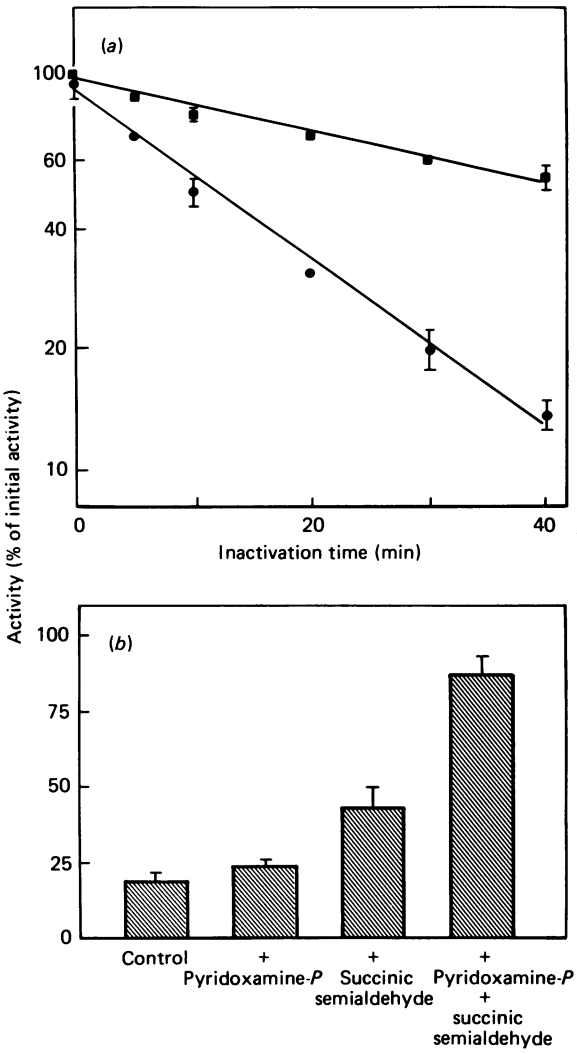


Fig. 4. Retardation of glutamate-promoted inactivation of glutamate decarboxylase by succinic semialdehyde and pyridoxamine 5′-phosphate

(*a*) The β-form of glutamate decarboxylase was preincubated with 10 mM-glutamate (●) or 10 mM-glutamate and 1 mM-succinic semialdehyde (■) for the indicated times at 30 °C in 50 mM-Hepes buffer, pH 7.2, containing 1 mM-2-aminoethylisothiouronium bromide. DL-[1-^{14}C]Glutamate was then added and the remaining activity determined in a 5 min assay. (*b*) The effect of 50 μM-pyridoxamine 5′-phosphate and 500 μM-succinic semialdehyde alone or together on inactivation by 10 mM-glutamate during a 30 min preincubation. Reaction conditions were the same as in (*a*).

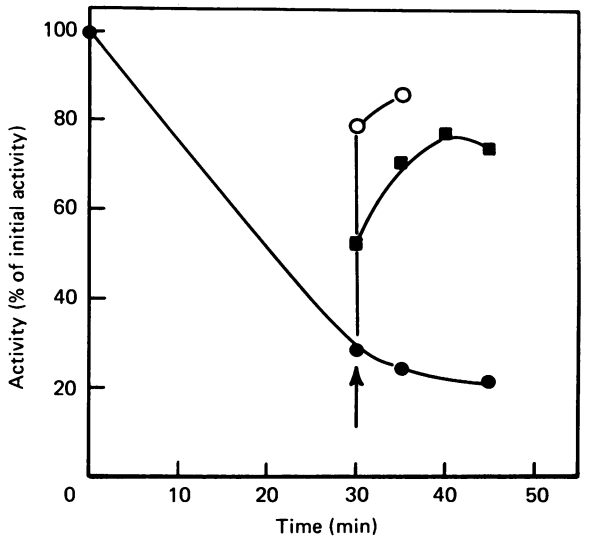


Fig. 5. Re-activation of glutamate decarboxylase apoenzyme by succinic semialdehyde and pyridoxamine 5′-phosphate

Apoenzyme was formed by preincubating the β-form of glutamate decarboxylase with 10 mM-glutamate, at 30 °C, in 50 mM-Hepes buffer, pH 7.2, containing 50 μM-pyridoxamine 5′-phosphate and 1 mM-2-aminoethylisothiouronium bromide (●). After 30 min, the re-activation period was begun (arrow) by adding 500 μM-succinic semialdehyde (■) or 50 μM-pyridoxal 5′-phosphate (○). The enzyme activity was measured in a 5 min assay at the indicated time.

effect on the rate of inactivation but enhanced the effect of succinic semialdehyde (Fig. 4*b*); 50 μM-pyridoxamine 5′-phosphate and 500 μM-succinic semialdehyde decreased the rate of inactivation 6-fold when added together. Similar effects of succinic semialdehyde and pyridoxamine 5′-phosphate were observed on 4-aminobutyrate-promoted inactivation of the enzyme.

Re-activation of the apoenzyme was studied by adding succinic semialdehyde to enzyme that had been inactivated by preincubation with 10 mM-glutamate in the presence of 50 μM-pyridoxamine 5′-phosphate (Fig. 5). On addition of 500 μM-succinic semialdehyde there was a time-dependent recovery of activity similar to that obtained by direct re-activation with pyridoxal 5′-phosphate. The presence of both pyridoxamine 5′-phosphate and succinic semialdehyde was required for re-activation; neither compound stimulated re-activation in the absence of the other. The transmission frequency of glutamate (as a percentage of decarboxylations) varied with pH and was completely paralleled by a change in the rate constants of inactivation across the range pH 6.5–8.5 (Fig. 6*a*). Similarly, the change in the rate constants of inactivation by 4-aminobutyrate exactly paralleled the extent of transamination represented by pyridoxamine 5′-phosphate production (Fig. 6*b*). In both cases, transamination and inactivation reach a maximum at around pH 8.0. The observed decreases in the transamination of and rate of inactivation by 4-aminobutyrate as compared with glutamate at the extremes of the pH range can be attributed to a decreased saturation of the enzyme by 4-aminobutyrate.

Frequencies of transamination catalysed by the α-, β- and γ-forms of glutamate decarboxylase

Quantification of the products of decarboxylation and transamination during the reaction of each form of glutamate decarboxylase with pyridoxal 5′-phosphate and glutamate is presented in Table 1. The measured ratio of pyridoxamine 5′-phosphate to succinic semialdehyde was almost exactly 1:1 for each form of the enzyme. The frequency of transaminations per decarboxylation, determined by quantifying both 4-aminobutyrate and pyridoxamine 5′-phosphate, was significantly different for each form. Thus at pH 7.2 the α-, β- and γ-forms catalysed only 1 transamination per 12000, 1 per 8500

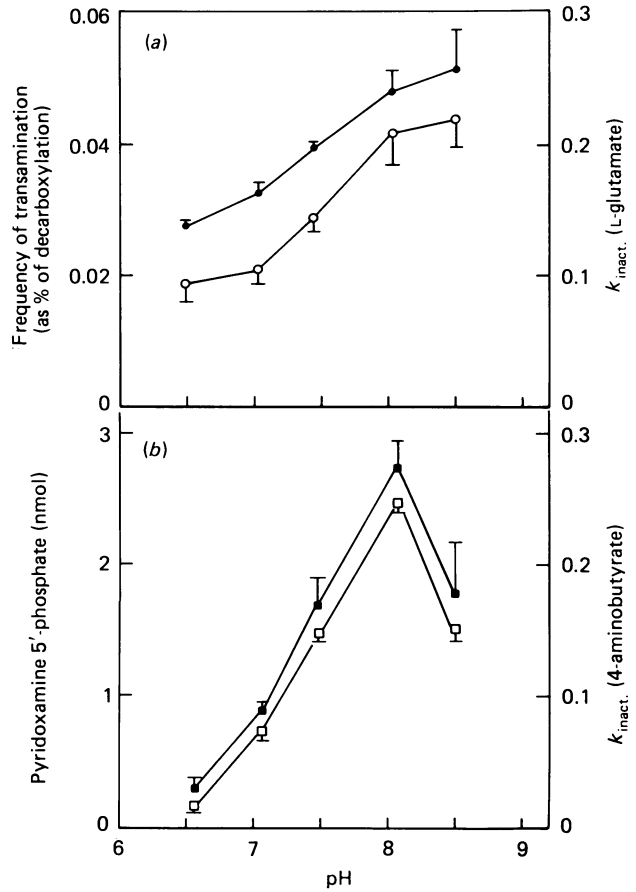


Fig. 6. Transamination of glutamate and 4-aminobutyrate by and rate constants of inactivation of glutamate decarboxylase as a function of pH

(*a*) ●, The frequency of decarboxylation-dependent transamination of glutamate by glutamate decarboxylase determined by quantifying 4-aminobutyrate and pyridoxamine 5′-phosphate production during a 2 h incubation and expressed as the percentage of decarboxylations. ○, Rate constants for inactivation by glutamate. (*b*) ■, The rate of transamination of 4-aminobutyrate determined by measuring pyridoxamine 5′-phosphate production. □, The rate constants for inactivation by 4-aminobutyrate. The concentrations of glutamate and 4-aminobutyrate were 20 and 200 mM respectively. Except for substrates, the reaction conditions (γ-form of glutamate decarboxylase, 30 °C, 75 mM-Hepes or Bicine buffer) were the same for all reactions.

and 1 per 3500 decarboxylations respectively. The values of these ratios were the same when decarboxylation was determined directly by measuring CO_2 release.

DISCUSSION

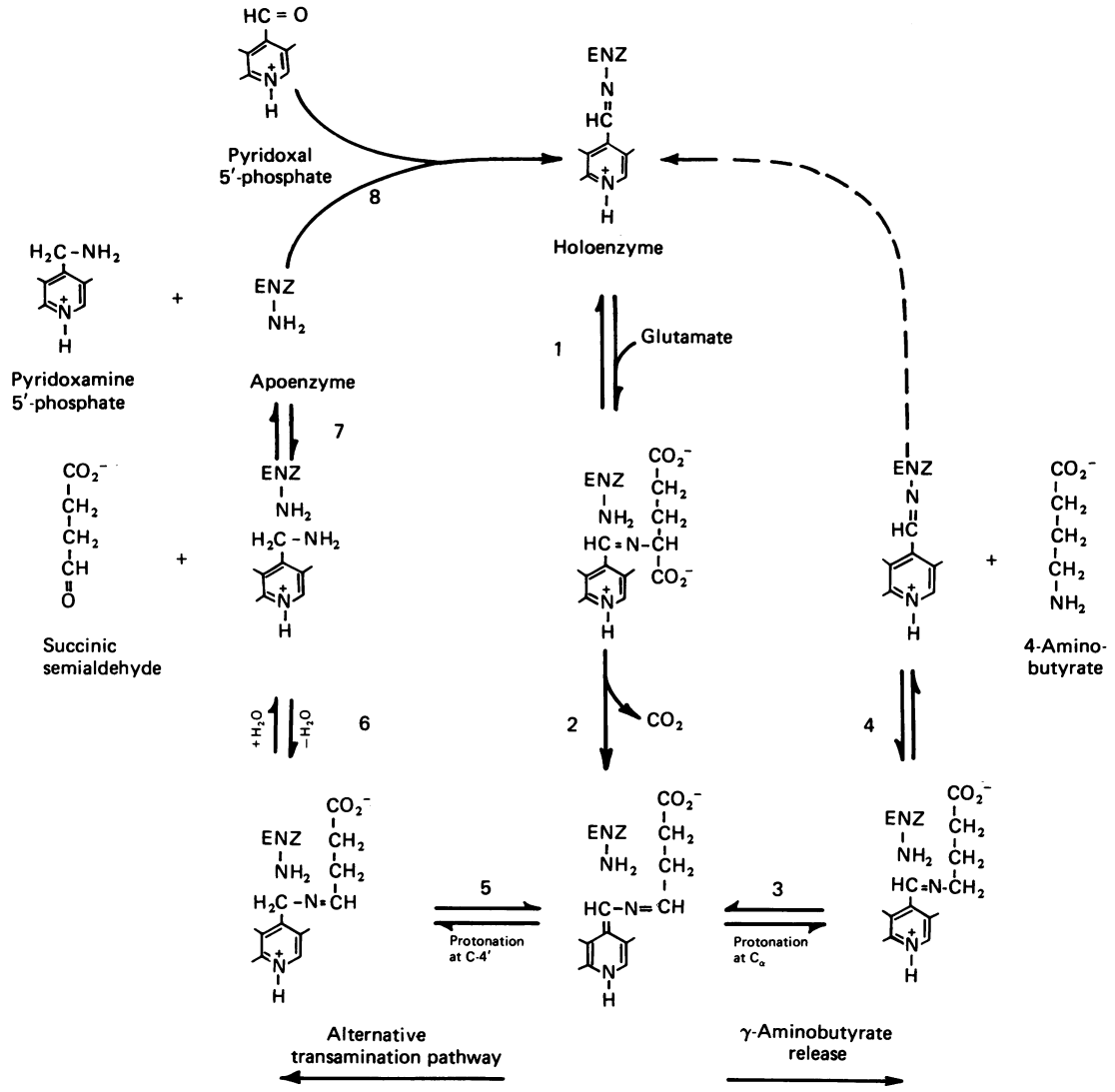
A mechanism of brain glutamate decarboxylase consistent with the experimentally obtained data is presented in Scheme 1. Initially glutamate binds to pyridoxal 5′-phosphate at the active site and the resulting Schiff base is decarboxylated (steps 1 and 2). The quinoid intermediate is then normally protonated on the carbon atom derived from the α-carbon of glutamate (C_α), resulting in release of 4-aminobutyrate and regeneration of holoenzyme (steps 3 and 4). Alternatively, protonation at the C-4′ carbon of the coenzyme converts the quinoid intermediate into pyridoxamine 5′-phosphate and succinic semialdehyde (steps 5 and 6). Dissociation of pyridoxamine 5′-phosphate from the active site (step 7) generates apoenzyme, which is reconstituted to holoenzyme by reaction with free pyridoxal 5′-phosphate (step 8).

Our results provide strong evidence for this mechanism. Direct conversion of pyridoxal 5′-phosphate into pyridoxamine 5′-phosphate during incubation of the enzyme with glutamate and pyridoxal 5′-phosphate was demonstrated by absorbance and fluorescence spectroscopy and by h.p.l.c. Production of succinic semialdehyde during the enzyme reaction was also demonstrated by h.p.l.c., and the stoichiometric ratio of pyridoxamine 5′-phosphate to succinic semialdehyde was found to be 1:1 as predicted by the mechanism. In addition, several other reactions were predicted from the mechanism and shown to occur. First, the enzyme was inactivated by incubation with 4-aminobutyrate in the absence of pyridoxal 5′-phosphate, a result consistent with reaction of 4-aminobutyrate with holoenzyme to form the quinoid intermediate followed by transamination to produce apoenzyme. Secondly, succinic semialdehyde and pyridoxamine 5′-phosphate were produced by incubating the enzyme with 4-aminobutyrate and pyridoxal 5′-phosphate. Under these conditions, the complete cycle of apoenzyme–holoenzyme interconversion could occur, allowing net production of succinic semialdehyde and pyridoxamine 5′-phosphate. Thirdly, addition of succinic semialdehyde retarded inactivation by glutamate in the absence of pyridoxal 5′-phosphate. This result is consistent with reaction of succinic semialdehyde with the enzyme–pyridoxamine 5′-phosphate complex. This retardation by succinic semialdehyde was enhanced by pyridoxamine 5′-phosphate, which

Table 1. Stoichiometry of the transamination products and the frequency of transamination catalysed by the multiple forms of glutamate decarboxylase

Each enzyme form was incubated with 20 mM-glutamate and 10 μM-pyridoxal 5′-phosphate for 3 h at 30 °C, and measurements were made as described in the Experimental section.

Enzyme form	Pyridoxamine-*P* (nmol)	Succinic semialdehyde (nmol)	4-Aminobutyrate (μmol)	Frequency of transamination: [Pyridoxamine-*P*] / [Succinic semialdehyde]	Frequency of transamination: [Pyridoxamine-*P*] × 100 / [4-Aminobutyrate]	Frequency of transamination: [Pyridoxamine-*P*] × 100 / [CO_2]
α	0.65±0.05	0.67±0.06	8.32±0.35	0.96	0.008	0.009
β	1.64±0.05	1.70±0.12	13.94±0.73	0.97	0.012	0.012
γ	4.87±0.14	4.54±0.37	17.54±0.84	1.07	0.028	0.028



Scheme 1. Proposed catalytic cycle of brain glutamate decarboxylase including the alternative transamination pathway

undoubtedly served to increase the amount of the enzyme–pyridoxamine 5′-phosphate complex. Pyridoxamine 5′-phosphate alone did not retard inactivation, indicating that negligible amounts of enzyme–succinic semialdehyde complex are formed and suggesting that succinic semialdehyde is released before pyridoxamine 5′-phosphate. Fourthly, succinic semialdehyde and pyridoxamine 5′-phosphate re-activated the apoenzyme, undoubtedly by reversing the transamination pathway to produce the quinoid intermediate followed by conversion into holoenzyme and 4-aminobutyrate.

The demonstration of a 1:1 stoichiometric ratio for the production of pyridoxamine 5′-phosphate and the aldehyde has not been previously reported for any other decarboxylase and provides strong evidence for decarboxylation-dependent transamination. We found no evidence for other amino-group acceptors on glutamate decarboxylase, such as those suggested for L-aromatic amino acid decarboxylase (Barboni *et al.*, 1981). The low transamination frequency catalysed by glutamate decarboxylase (< 0.05% of decarboxylations) is similar to the values reported for other pyridoxal 5′-phosphate-dependent amino acid decarboxylases acting on their natural substrates (O'Leary & Baughn, 1977). In addition, the present study demonstrates several reactions catalysed by brain glutamate decarboxylase that may also be common to other pyridoxal 5′-phosphate-dependent α-decarboxylases. The activation by succinic semialdehyde appears similar to the activation of aspartate β-decarboxylase by α-oxo acids (Novogrodsky & Meister, 1964). However, the requirement for free pyridoxamine 5′-phosphate to achieve effective activation suggests that brain glutamate decarboxylase has a low affinity for pyridoxamine 5′-phosphate. The production of succinic semialdehyde from 4-aminobutyrate appears to be the first demonstration of a direct transamination of an amino acid catalysed by an α-decarboxylase.

The similarity of the rates of pyridoxamine 5′-phosphate production in the presence of saturating concentration of glutamate and 4-aminobutyrate is consistent with the similarity in the rates of inactivation of the enzyme by the two amino acids (Spink *et al.*, 1985). These similarities also suggest that there is a slow step in the reaction mechanism after the decarboxylation step. If hydrolysis

of the quinoid intermediate to release 4-aminobutyrate were much faster than decarboxylation, the concentration of the quinoid intermediate generated by incubation with saturating glutamate would be low, since it would be immediately hydrolysed (steps 3 and 4). In contrast, high concentrations of the intermediate would be formed rapidly during incubation with 4-aminobutyrate, since the transamination pathway (steps 5–8) is clearly much slower than the hydrolytic reactions (steps 3 and 4). Thus if decarboxylation were rate-limiting the maximum rates of inactivation and transamination would be expected to be lower with glutamate than with 4-aminobutyrate, since these rates depend directly on the concentration of the intermediate. The similar rates of inactivation observed with glutamate and 4-aminobutyrate are inconsistent with the rate-limiting step being decarboxylation, and are consistent with a slow step after decarboxylation. This interpretation is consistent with carbon-isotope studies of other decarboxylases where, for the natural substrate, neither decarboxylation nor Schiff-base interchange appeared to be entirely rate-limiting (O'Leary, 1977; O'Leary & Piazza, 1978).

Decarboxylation-dependent transamination and transamination of 4-aminobutyrate were optimal close to pH 8.0, a clearly different value from the optimum for decarboxylation, which was close to pH 6.5 (Spink *et al.*, 1985). There was a direct relationship between the rate of transamination as measured by production of pyridoxamine 5′-phosphate and the magnitude of the rate constants of inactivation by glutamate and 4-aminobutyrate across the entire pH range. This would be expected if inactivation is a result of transamination, and implies that the transamination pathway is rate-limiting in the inactivation–re-activation cycle. This interpretation is also supported by the observation that the rate constants for activation (Spink *et al.*, 1985) are greater than the rate constants for inactivation. Further, the parallel change in transamination frequency and inactivation rates as a function of pH provided convincing evidence that decarboxylation and transamination were the activities of the same active site.

All three molecular forms of glutamate decarboxylase were shown to catalyse decarboxylation-dependent transamination of glutamate. In each case the stoichiometric ratio of pyridoxamine 5′-phosphate to succinic semialdehyde was 1:1. However, the transamination frequency differed considerably among the forms (γ-form > β-form > α-form). This order is the same as the order of inactivation of each form by glutamate in the absence of cofactor (Spink *et al.*, 1985), providing further evidence that inactivation results from the transamination mechanism. The results also suggest that the active sites of the three forms differ in a way that alters the relative rates of protonation at C-4′ and $C_{(\alpha)}$ of the quinoid intermediate. From hydrogen-isotope discrimination experiments there is evidence that a single monotropic catalytic group acts as the proton source for protonation of the quinoid intermediate during decarboxylation of L-glutamate by bacterial glutamate decarboxylase (Yamada & O'Leary, 1977). However, whether the proton source for transamination is the same group, a neighbouring group or the solvent is unknown. Thus the different transamination frequencies of the three forms of brain decarboxylase may be explained by an altered orientation of one or more proton-donating groups at the active site or, alternatively, by differences in the shielding of C-4′ from solvent.

The results of this study and the accompanying paper (Spink *et al.*, 1985) indicate that the α-, β- and γ-forms of glutamate decarboxylase from pig brain each catalyse decarboxylation-dependent transamination of L-glutamate and transamination of 4-aminobutyrate, and in the presence of pyridoxal 5′-phosphate there is a complete cycle of inactivation (apoenzyme formation) and re-activation (holoenzyme formation). The rates of inactivation and re-activation are different for each form of the enzyme, and result in differences in cofactor saturation among the three forms. These differences suggest that the three forms of glutamate decarboxylase catalyse 4-aminobutyrate synthesis at substantially different rates *in vivo*, depending on physiological conditions.

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