Kinetic and Spectral Properties of Rabbit Brain 4-Aminobutyrate Aminotransferase

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Purification of 4-aminobutyrate–2-oxoglutarate aminotransferase (EC 2.6.1.19) from rabbit brain is described. The method was used as a routine to give between 5 and 10 mg of pure enzyme from 750 g of rabbit brain. The enzyme is a dimer made up of subunits each with a mol.wt. of 58000. An absorption spectrum of the freshly prepared enzyme shows peaks at 415 and 330 nm. Treatment of the enzyme with the substrate 4-aminobutyrate or glutamate produces a decrease in the 415 nm and an increase in the 330 nm peak. This conversion, which is attributed to an aldimine into ketimine step in the reaction, is sufficiently slow when 4-aminobutyrate is the substrate to allow it to be followed by stopped-flow spectrophotometry. A first-order rate constant was determined for this step (12 s^{-1}) and compared with the turnover number for the enzyme derived by steady-state methods (9.5 s^{-1}). The first-order rate constant when glutamate was used as substrate was estimated to be approx. 30 s^{-1} .

The enzyme 4-aminobutyrate–2-oxoglutarate aminotransferase (EC 2.6.1.19) is interesting to us for two reasons. From a pharmacological point of view, our interest in the enzyme stems from the physiological effects produced by a deliberate decrease of its activity after specific chemical modification *in vivo* (Fowler & John, 1972; Fowler, 1973; Baxter *et al.*, 1973). These effects are probably due to the influence of the enzyme on the metabolic concentration of one of its substrates, the neurotransmitter 4-aminobutyrate. From an enzymological point of view we find it interesting partly because of its low catalytic rate constant. This makes spectral transitions caused by substrate-induced chemical changes in the coenzyme pyridoxal phosphate accessible to rapid-mixing techniques.

Convincing reports of homogeneous preparations of this enzyme from brain of two species have appeared. Vasil'ev & Eremin (1968) obtained a preparation from rat brain of sufficient purity and in sufficient quantity to allow coenzyme spectra to be determined, and Maitre *et al.* (1975) have purified 0.5 mg of the enzyme from the same source. Schousboe *et al.* (1973, 1974) have purified the enzyme from mouse brain and, on the basis of polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate, claimed the enzyme to be a dimer with subunits of different molecular weight. A third report (Bloch-Tardy *et al.*, 1974) has also been published of a purification of the enzyme from pig brain.

Our long-term objective is to relate those aspects of physiological behaviour that depend on this enzyme to events occurring at a molecular level. We therefore wished to purify the enzyme from a relatively small animal, but at the same time obtain sufficient tissue to allow preparation of reasonably large quantities of the enzyme. Rabbit brain was chosen as a source of the enzyme, since it is available as a by-product from rabbits produced on a large scale for the table.

Experimental

Chemicals

Pyridoxal 5'-phosphate, 2-oxoglutaric acid, 3,5-diaminobenzoic acid, bovine serum albumin and 4-aminobutyrate were bought from Sigma (London) Chemical Co., London S.W.6, U.K. Other chemicals used were supplied by BDH Chemicals Ltd., Poole, Dorset, U.K. The enzymes used as standards for molecular-weight determinations were bought from Boehringer, London W.5, U.K.

Column chromatography

Ion-exchange celluloses (microgranular grades CM-52 and DE-52) were obtained from Whatman Biochemicals Ltd., Maidstone, Kent, U.K. Linear concentration gradients were produced by using two cylindrical vessels of the same size connected at the bottom by a 5 mm polythene tube.

Electrophoresis

Starch-gel electrophoresis was carried out as described by Barrett *et al.* (1962). Polyacrylamide-gel electrophoresis in sodium dodecyl sulphate was carried out after reduction with 2-mercaptoethanol as described by Weber & Osborn (1969).

Enzyme and protein assays

Routine assays of the enzyme, as well as the determination of steady-state parameters, were carried out by stopping the initial enzymic reaction by the addition of trichloroacetic acid to a concentration of 10% (w/v). The concentration of succinic semialdehyde formed was determined with 3,4-diaminobenzoic acid by the method of Salvador & Albers (1959). For routine assays during purification, the enzymic reaction carried out was in 30mM-4-aminobutyrate and 12.5mM-2-oxoglutarate in 50mM-boric acid, adjusted to pH 8.4 with NaOH, and at 37°C. The determinations of the steady-state parameters are given in the appropriate Figure legends. Protein concentrations were measured by the method of Lowry *et al.* (1951). The moisture content of the standard, bovine serum albumin, was found to be 2.5%. This was determined by loss of weight on drying over P₂O₅ *in vacuo* at 20°C for 24 h.

Stopped-flow measurements

Measurement of the rates of change in the absorption spectrum of enzyme-bound coenzyme on mixing with substrate were made by using a Durrum-Gibson stopped-flow spectrophotometer. The output from the photomultiplier of this instrument was displayed on a Tektronix type RM 564 storage oscilloscope.

Enzyme preparation

Rabbit brain was obtained from the Buxted-Olac Co., Buxted, Sussex, U.K. The brains were removed as soon as possible after death, and stored frozen at -20°C until required. For the sake of convenience, the enzyme was purified from 750g batches of the frozen brain. Enzyme-preparation procedures were carried out generally at 4°C. A Waring Blendor was used to prepare a 25% (w/v) homogenate in a solution (at pH 7.0) which contained 10mM-sodium phosphate buffer, 1mM-EDTA, 0.1m-pyridoxal phosphate, 0.1% (v/v) 2-mercaptoethanol, 1mM-2-oxoglutarate and 0.5% (v/v) Triton X-100. The homogenate was adjusted to pH 5.3 with 2% (v/v) formic acid, heated to 53°C for 5 min and, after cooling to 5°C, centrifuged at 5000g for 30 min. The supernatant was then treated with (NH₄)₂SO₄; the precipitate obtained at 45–65% saturation was dissolved in a buffer identical with that used in the initial homogenization,

except that the Triton X-100 was omitted. It was then dialysed against 10mM-HCl containing 10mM-sodium acetate, adjusted to pH 7.5 with solid Tris, and applied to a column (15cm×4cm) of DEAE-cellulose equilibrated with the same buffer. The column was eluted with a linear gradient starting with the equilibration buffer, and with an increase in the sodium acetate concentration to 50mM over a volume of 500ml. The enzyme was eluted at about 350ml, and was dialysed against 40mM-sodium acetate, which had been adjusted to pH 5.4 with acetic acid. It was then chromatographed on a column (10cm×1cm) of CM-cellulose equilibrated in the same buffer. The enzyme was eluted in about 250ml by using a linear gradient made with the equilibration buffer (250ml) and the same volume of 80mM-sodium acetate, also adjusted to pH 5.4 with acetic acid. The results from one such purification are shown in Table 1.

The losses at the (NH₄)₂SO₄ stage can be fully accounted for in the discarded precipitate and supernatant. However, the yields shown for the two column steps are total yields, since all of the active fractions were pooled.

Occasionally, when starch-gel electrophoresis showed impurities, the enzyme was further purified by chromatography on a column (5cm×1cm) of DEAE-cellulose under the conditions described above. At this stage the elution of the enzyme could be followed by absorption at 415nm. The pure enzyme was kept in 50mM-sodium acetate buffer, pH 5.4, and in this solution could be kept at 4°C for several weeks without loss of activity. The method as a routine gives between 5 and 10mg of pure enzyme.

Results and Discussion

Homogeneity, molecular weight and subunit composition

Plate 1(a) shows that the enzyme preparation is homogeneous on starch-gel electrophoresis. Plate 1(b) shows polyacrylamide-gel electrophoresis of the enzyme in sodium dodecyl sulphate. The subunit mol.wt. was estimated to be 58000 when using this gel and six proteins of known molecular weight which were run simultaneously on separate gels. The six proteins used as standards in the determination were (with mol.wts. in parentheses) β -galactosidase from *Escherichia coli* (130000), bovine serum albumin (68000), beef liver catalase (60000), rabbit muscle aldolase (40000), bovine pancreatic chymotrypsinogen (25700) and horse heart cytochrome *c* (12500). The present preparation gives a single band of molecular weight corresponding to that of the larger of the two subunits reported by Schousboe *et al.* (1974), and there was no evidence of the smaller subunit found by these workers; perhaps the smaller

EXPLANATION OF PLATE I

Electrophoresis of the purified enzyme

The photographs show starch-gel electrophoresis of the enzyme (a) and polyacrylamide-gel electrophoresis of the enzyme in sodium dodecyl sulphate (b).

Table 1. *Purification of 4-aminobutyrate aminotransferase from rabbit brain*

Details of each step in the purification are given in the text. A unit of enzyme activity is defined as that amount of enzyme which produces 1 $\mu\text{mol/min}$ of succinic semialdehyde when using the routine assay conditions described in the text. The yields quoted in the last column represent the amount taken for the next step in the purification.

Treatment	Volume (ml)	Protein (mg/ml)	Sp. activity (units/mg at 37°C)	Yield (%)
Homogenate	2850	26	0.0148	100
Supernatant after pH 5.3 and 53°C	1750	4.3	0.078	48.9
(NH_4) ₂ SO ₄ (45–65% satn.)	274	4.6	0.414	47.7
DEAE-cellulose (0.01 M–0.05 M-sodium acetate/Tris, pH 7.5)	158	0.7	1.97	20
CM-cellulose (0.04 M–0.08 M-sodium acetate, pH 5.4)	60	0.1	17.3	9.5

Fig. 1. *Spectral changes brought about by treatment of the enzyme with amino substrates*

The spectrum with the highest absorbance at 415 nm and lowest absorbance at 330 nm is that of the freshly isolated enzyme (Tris/HCl buffer; $[\text{Cl}^-] = 0.1 \text{ M}$; pH 7.5). Successive additions of amino acid substrates (0.25, 2.0, 5.75 and 20.8 mM-4-aminobutyrate and then additional 20, 120 and 243 mM-glutamate) led to the progressive decrease in absorption at 415 nm and the increase shown at 330 nm. Spectra were recorded using 1 cm-light-path quartz cuvettes in a Pye–Unicam SP.800 spectrophotometer.

subunit they claimed to be an integral part of the native enzyme resulted from proteolytic degradation of the 58000-mol.wt. subunit.

When the enzyme used in the present study was chromatographed on Sephadex G-200 in 0.1 M-Tris/HCl buffer, it ran as a single peak with enzyme activity coinciding with protein concentration estimated by E_{280} . The method of Andrews (1964) was used to calibrate the same column with standard proteins (rabbit muscle aldolase, mol.wt. 158000; pig heart lactate dehydrogenase, 140000; pig heart aspartate aminotransferase, 92000; bovine haemoglobin, 64500; and cytochrome *c*, 12500) and the mol.wt. of the 4-aminobutyrate amino transferase was determined to be 120000 ± 10000 . We conclude therefore that the enzyme is a dimer composed of subunits with identical molecular weights.

Spectral properties

Fig. 1 shows absorption spectra of the enzyme. The spectrum with the highest peak at 415 nm and lowest peak at 330 nm is that of the enzyme obtained at the end of the preparation, and without further treatment. The remaining spectra were obtained by making successive additions of amino acid substrates. By analogy with other aminotransferases [see the review by Fasella (1967)], this spectral change represents the conversion of the aldimine enzyme absorbing at 415 nm into ketimine and free amine forms of the enzyme, both of which would be expected to absorb in the region of 330 nm. Dialysis of the enzyme after treatment shown in Fig. 1 left the spectrum unchanged until the addition of 2-oxo-glutarate, which reversed the change restoring the

Fig. 2. Reaction of 4-aminobutyrate aminotransferase with amino acid substrates

Samples of the enzyme were treated with (a) 4-aminobutyrate and (b) glutamate. Both reactions were carried out in Tris/HCl buffer ($[Cl^-] = 0.1M$; pH 7.5). Enzyme concentrations were 1.5 mg/ml (a) and 2.0 mg/ml (b). The graphs show ΔE_{415} .

original spectrum. The dependence of absorbance at 415 nm on 4-aminobutyrate and glutamate concentration is shown in Fig. 2.

The spectrum of the enzyme is unaltered by pH changes in the range 5.5–9.0, and the form absorbing at 415 nm is clearly an active enzyme. In both these respects, 4-aminobutyrate aminotransferase differs from aspartate aminotransferase and alanine amino-

transferase, both of which absorb in the 410–430 nm region only at acid pH values, where they are inactive (Jenkins *et al.*, 1959; Saier & Jenkins, 1967). At least three other aminotransferases have spectral properties very similar to 4-aminobutyrate aminotransferase. The enzymes transaminating D-alanine, L-leucine and L-ornithine (Martinez-Carrion & Jenkins, 1965; Taylor & Jenkins, 1966; Peraino *et al.*, 1969) all have pH-independent absorption maxima at about 415 nm, and are enzymically active in this form.

There is, however, a clear peak at 330 nm in the freshly prepared enzyme, and additions of 2-oxoglutarate to the enzyme in this state did not alter the spectrum. This peak cannot therefore be ascribed to pyridoxamine enzyme. Since the spectrum is also unchanged by alterations in pH, the 330 nm species cannot be derived from the 415 nm species by simple deprotonation. An absorption peak is also present in this part of the spectrum in all of the enzymes mentioned above, and in the case of aspartate aminotransferase it has been convincingly ascribed to binding of the coenzyme in an inactive mode (Martinez-Carrion *et al.*, 1967). It would, however, be unsafe at this stage to conclude that 4-aminobutyrate aminotransferase contains coenzyme bound in an analogous inactive way, since an absorption band in this region of the spectrum is characteristic of many forms of pyridoxal phosphate (Johnson & Metzler, 1970).

Kinetic properties

Pure preparations of 4-aminobutyrate aminotransferase obtained from other species (Vasil'ev & Eremin, 1968; Schousboe *et al.*, 1974) have had relatively low specific activities of approx. $5 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$, suggesting either that the mechanism includes a slow step or that the preparations obtained contained substantial amounts of inactive protein. In the present work, the turnover number (k_{cat}) was determined by steady-state kinetic methods described by Velick & Vavra (1962) using a subunit mol.wt. of 58000, and protein concentration was measured by the method of Lowry *et al.* (1951). A rate constant (k_a) for the rate-limiting step in the reaction between the aldimine enzyme and 4-aminobutyrate was measured in a stopped-flow spectrophotometer and a single turnover of the enzyme at different substrate concentrations was observed. These rate constants should be very nearly the same if all of the enzyme is fully active, and if a definite rate-limiting step of the overall transamination occurs in this half of the reaction.

Fig. 3 shows a secondary plot used to derive k_{cat} . It is essentially the same as that used by Velick & Vavra (1962), except that $1/v$ has been converted into $1/k_{\text{obs}}$ by allowing for molecular weight and protein

concentration. The value obtained for k_{cat} was $9.5s^{-1}$. K_m values for 4-aminobutyrate and 2-oxoglutarate were 18 and 5mM respectively. It should be noted that all the kinetic determinations were made in 50mM-acetate (50mM-acetic acid adjusted to pH8.2 with solid Tris), and that acetate binds to the enzyme with a dissociation constant of 57mM (L. J. Fowler, unpublished results).

Fig. 4 shows photographs of oscilloscope traces obtained when 4-aminobutyrate was mixed with the enzyme. Fig. 4(a) shows increasing transmission at 410nm and Fig. 4(b) shows decreasing transmission at 330nm. Both curves can be described by a single exponential process with an apparent rate constant (k_{app}) $11s^{-1}$. The speed of the process was found to be dependent on substrate concentration, and Fig. 5 shows a plot of $1/k_{app}$ against $1/[4\text{-aminobutyrate}]$. The linearity of this plot is consistent with a simple scheme in which initial, rapidly reversible binding is followed by rate-limiting

tautomerization of aldimine into ketimine, since for such a scheme:

$$\frac{1}{k_{app}} = \frac{K_s}{k_a} \cdot \frac{1}{[S]} + \frac{1}{k_a}$$

From Fig. 5, k_a was determined to be $12s^{-1}$ and K_s to be 9mM. The similarity between the rate constants determined by these two methods shows that most, if not all the enzyme is fully active, and that the slowest step in the overall transamination in this direction is in the half-reaction in which 4-aminobutyrate is converted into succinic semialdehyde. The difference between the values obtained for k_{cat} and k_a could easily be due to experimental error, particularly since k_{cat} relies on knowledge of the molarity of the enzyme used as well as on steady-state kinetic measurements. However k_{cat} will of course be lower than k_a if there is a step in the other

Fig. 3. Determination of steady-state parameters

Velocities were measured as described in the Experimental section. The enzyme assays were carried out at $25^{\circ}C$, and the results obtained were first plotted as $1/v$ against $1/[S]$ for one substrate, with different fixed concentrations of the other substrate, to give two sets of parallel lines indicating a Ping Pong mechanism. These lines were analysed for their coefficients of linear regression and intercept values. The values for V_{max} determined in this way were converted into k_{obs} by dividing by the molarity of the enzyme used and are here plotted against reciprocal substrate concentration to give k_{cat} .

Fig. 4. Reaction of aldimine enzyme with 4-aminobutyrate (a) Oscilloscope trace showing increasing transmission at 410nm when 4-aminobutyrate (40mM) was mixed with the aminotransferase (2mg/ml); (b) same reaction followed at 330nm. The reaction was carried out at $25^{\circ}C$ and time-constants for the 415nm trace and 330nm trace were 1 and 5ms respectively.

Fig. 5. Determination of k_a from stopped-flow experiments

Each point represents a first-order rate constant ($k_{app.}$) derived from the exponential process observed at 410nm when 4-aminobutyrate at the concentrations indicated was mixed with the enzyme (2mg/ml). The reactions were carried out at 25°C.

half-reaction (oxoglutarate into glutamate) which is also rate-limiting.

In comparison with other transamination reactions, the turnover of this enzyme is unusually slow. Fasella & Hammes (1967) reported a value of 370s^{-1} for aspartate aminotransferase in the direction aspartate + 2-oxoglutarate $\rightarrow$ glutamate + oxaloacetate, and 920s^{-1} in the reverse direction. These turnover numbers were calculated from the rate-limiting rate constants observed for each half of the reaction. For example the 370s^{-1} rate constant is largely the result of a 530s^{-1} constant for the aspartate-into-oxaloacetate half-reaction and 1330s^{-1} rate constant for the 2-oxoglutarate-into-glutamate half-reaction. The speed of these reactions makes them virtually inaccessible to rapid-mixing techniques (Gutfreund *et al.*, 1961). Saier & Jenkins (1967) found L-alanine aminotransferase to have a turnover number of 1100s^{-1} . Although we are unable to find accurate turnover numbers for other aminotransferases, we estimate from specific activities and other relevant published data (Taylor & Jenkins, 1966; Martinez-Carrion & Jenkins, 1965) that both L-leucine and D-alanine aminotransferases must also be at least ten times faster than 4-aminobutyrate aminotransferase. It is possible that transamination of 4-aminobutyrate is slow because the amino group transferred is far removed from the carboxyl group which, in other reactions, might be expected to influence the loss of proton from the amino group bearing carbon. This point was examined by using

glutamate instead of 4-aminobutyrate as amino acid substrate. The first-order rate constant for increase in transmission at 410nm was found to have a hyperbolic dependence on glutamate concentration similar to that found for 4-aminobutyrate. A double-reciprocal plot gave a value of 30s^{-1} for k_a and 20mM for K_s .

The fundamental catalytic step of transamination is considered to be the loss of hydrogen from the carbon atom that bears the amino group (Braunstein, 1960; Guirard & Snell, 1964). This step is rate-limiting in the transamination of aspartate (Banks *et al.*, 1968). It seems likely, therefore, that 4-aminobutyrate is transaminated more slowly than glutamate, because it lacks the electron-withdrawing effect of the carboxyl group on the appropriate carbon atom.

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