Purification and partial characterization of 4-aminobutyrate:2-oxoglutarate aminotransferase from sheep brain and locust ganglia

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We report here the first purification to homogeneity of 4-aminobutyrate:2-oxoglutarate aminotransferase (EC 2.6.1.19) (GABA-T) from an invertebrate source (locust) and its initial comparison with that of GABA-T from mammalian brain (sheep). The enzyme from both organisms was found to be a dimer of similar-sized subunits, with a native M_r of approx. 97 000. The pI of GABA-T from the locust was 6.7 and that of the sheep enzyme was 5.5. Michaelis constants for 4-aminobutyric acid (GABA) and 2-oxoglutarate were respectively 0.79 ± 0.16 mM and 0.27 ± 0.08 mM for the locust enzyme and 2.2 ± 0.24 mM and 0.22 ± 0.11 mM for the sheep enzyme. 5-(Aminomethyl)-3-isoxazolol (muscimol) was a competitive inhibitor of both enzymes, whereas 5-amino-1,3-cyclohexadienylcarboxylic acid (gabaculine) acted as a potent suicide substrate. However, 3-aminopropane-1-sulphonic acid, diaminobutyric acid, 1,2,3,4-tetrahydro-1-methyl-3-pyridinecarboxylic acid (isoguvacine), β -(aminomethyl)-4-chlorobenzenepropanoic acid (baclofen), bicuculline and picrotoxin did not inhibit either enzyme at concentrations below 100 mM. Polyclonal antisera raised against GABA-T from the sheep failed to cross-react with the enzyme from locust in either an Ouchterlony immunodiffusion plate or a competitive enzyme-linked immunosorbent assay. The purification procedures differed considerably. Ion-exchange chromatography, which was found suitable for the purification of GABA-T from the sheep, was ineffective with locust enzyme, which was finally purified by hydrophobic-interaction chromatography and chromatofocusing.

INTRODUCTION

4-Aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the nervous system of vertebrates and invertebrates. In such tissues there are two separate proteins with which this small ligand interacts: the GABA receptor, a complex multimeric protein with binding sites for GABA, benzodiazepines and barbiturates [1]; and 4-aminobutyrate:2-oxoglutarate aminotransferase (GABA-T), the enzyme that inactivates GABA by conversion into succinic semialdehyde, which is then fed back into the citric acid cycle via the GABA shunt [2].

We are comparing these two proteins from the supraoesophageal ganglia of the locust *Schistocerca gregaria*. Since both proteins bind the same ligand, it is interesting from an evolutionary standpoint to ascertain whether they share common structural features. Thus it may be that the two proteins have a common ancestral genetic origin, but have diverged during evolution. Furthermore, if significant differences exist between the invertebrate proteins and their mammalian counterparts, then the possibility exists of synthesizing selective pesticides against either the receptor or the enzyme from the locust. This paper reports the purification and initial characterization of GABA-T from sheep and locust brain.

MATERIALS AND METHODS

Experimental material

Locusts were purchased from Larajon Locust Suppliers, c/o Welsh Mountain Zoo, Colwyn Bay, North Wales, U.K. Sheep brains were obtained fresh from a local abattoir.

Column chromatography

Ion-exchange pre-swollen microgranular celluloses were obtained from Whatman, Maidstone, Kent, U.K. Mono Q, Mono S, Mono P and Sephadex G-200 were supplied by Pharmacia, Milton Keynes, Bucks., U.K. AH (1,6-diaminohexane)- and CH (6-aminohexanoic acid)-Sepharose 4B were supplied by Sigma Chemical Co., Poole, Dorset, U.K.

Enzyme assays

GABA-T was assayed by a new method. The procedure relies on coupling GABA-T and succinic semialdehyde dehydrogenase (SSDH) and thereby oxidizing the succinic semialdehyde formed by GABA-T to succinate, with concomitant reduction of NADP⁺ to NADPH. The progress of the reaction may therefore be monitored spectrophotometrically at 340 nm. The coupling enzyme was prepared from the commercial preparation GABASE (Sigma Chemical Co.) by separation from

Abbreviations used: GABA, 4-aminobutyrate; GABA-T, 4-aminobutyrate:2-oxoglutarate aminotransferase (EC 2.6.1.19); SSDH, succinic semialdehyde dehydrogenase (EC 1.2.1.16).

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GABA-T by gel filtration on Sephadex G-25. This new coupled assay was tested thoroughly and shown to be a valid measure of GABA-T activity. The exact protocol is as follows: 3 mg of freeze-dried GABASE was resuspended in a mixture of 0.5 ml of 20 mM-Bis-tris-propane {1,3-bis[tris(hydroxymethyl)methylamino]propane}, pH 7.3, and 0.5 ml of glycerol. A 0.33 ml portion of the mixture was applied to a Pharmacia PD-10 column, which had been equilibrated in the same buffer. A small amount of Blue Dextran was added to the applied mixture; this facilitated the detection of protein, as Blue Dextran is eluted in the void volume. After elution from the PD-10 column, all GABA-T activity was lost, whereas SSDH remained completely active. The eluted SSDH was used as the linking enzyme when measuring GABA-T activity from the locust. If stored in 30% (v/v) glycerol/1 mM-dithiothreitol, the SSDH remained active for several weeks. The assay mixture contained the following: 50 mM-Bicine, pH 8.5, 3 mM-2-oxoglutarate, 0.2 mM-NADP⁺, 0.5 mM-dithiothreitol, extract, and 25 μ l of SSDH. The reaction was started by the addition of 6 mM-GABA to give a final volume of 1 ml. The change in A_{340} is followed, and the unit of activity is defined as the amount of enzyme catalysing the reduction of 1 μ mol of NADP⁺/min under assay conditions.

Succinate dehydrogenase (EC 1.3.99.1) was assayed by the method of Muller *et al.* [3]. Michaelis constants were determined by the method of Eisenthal & Cornish-Bowden [4], by using a computer program available at Bath University.

Enzyme purification

GABA-T from sheep brain was purified by the method of John & Fowler [5], with the following minor amendments: the resuspension and DEAE-52 equilibration buffers were 20 mM-Tris/acetate, not 10 mM, and the CM-52 gradient ran from 40 to 120 mM-sodium acetate, not 40–80 mM.

GABA-T from locust ganglia was purified as follows: locusts were decapitated and their heads homogenized in a Waring blender containing 20 mM-sodium phosphate buffer, 100 μ M-pyridoxal phosphate, 4 mM-EGTA, 0.1% (v/v) 2-mercaptoethanol, 1 mM-2-oxoglutarate and 0.2% (v/v) Triton X-100, all at pH 7.5, at a buffer/tissue ratio of 3:1 (v/w). The homogenate was strained through four layers of muslin and the pH lowered to 5.4 by the addition of 2% (v/v) formic acid. The homogenate was heated to 55°C for 5 min and then cooled to 10°C before being centrifuged at 5000 *g* for 30 min. The supernatant was fractionated with (NH₄)₂SO₄; the pellet from the 45–65% satn. fraction was resuspended in the extraction buffer without Triton X-100, dialysed against a buffer containing 20 mM-sodium acetate and 20 mM-HCl adjusted to pH 8.0 with solid Tris, and then applied to a column (30 cm $\times$ 1.5 cm) of DEAE-cellulose, equilibrated with the same buffer. GABA-T was detected in the washings, which were dialysed against a solution containing 20 mM-Mops and 35% satn. (NH₄)₂SO₄, pH 6.8, and applied to a column of phenyl-agarose (12 cm $\times$ 1.5 cm) equilibrated with the same buffer. GABA-T was eluted with a decreasing (NH₄)₂SO₄ gradient from 35 to 15% satn. over 500 ml. Active fractions were concentrated to 1.5 ml in an Amicon ultrafiltration cell and dialysed against 25 mM-Bis-tris/HCl, pH 7.1, before being loaded on to the Mono P

column of the fast protein liquid chromatography system, equilibrated with the same buffer. The column was eluted at a flow rate of 0.5 ml/min with Polybuffer 74, pH 5.0, over a gradient of 40 ml.

Electrophoresis

Gel electrophoresis in the absence and presence of SDS was performed as described by Laemmli [6].

Immunological assays

The dot-immunobinding assay was performed as described by Hawkes *et al.* [7]. Immunodiffusion experiments were performed by the method of Ouchterlony [8], and competitive enzyme-linked immunosorbent assays by the method of Voller *et al.* [9].

Localization studies

Subcellular localization of GABA-T from locust supraoesophageal ganglia was performed as follows. Ganglia were removed with forceps after slicing the cuticle from the front of the head, and were then homogenized gently in a Dounce glass homogenizer containing 300 mM-sucrose, 2 mM-EDTA, 20 mM-Mops and bovine serum albumin (fatty-acid free) at 0.75 mg/ml, all adjusted to pH 8.0. The albumin was included in order to complex non-esterified fatty acids, which would otherwise disrupt the mitochondrial membranes. The homogenate was strained through four layers of muslin and centrifuged at 2500 *g* for 10 min, after which the supernatant was centrifuged at 10000 *g* for 30 min. The pellet was resuspended in a minimum of Percoll (Pharmacia) dilution buffer and fractionated on a Percoll gradient, by the method of Jeffery *et al.* [10].

RESULTS AND DISCUSSION

All attempts to purify GABA-T from locust ganglia by the fast-protein-liquid-chromatography method proved unsuccessful, because binding to the Mono Q column did not occur, even at pH 9; although the enzyme bound to Mono S at pH 5.4, no activity was detected upon elution. Affinity chromatography using gabaculine bound to AH- and CH-Sepharose was also ineffective as a means of purification. Although gabaculine bound to both types of Sepharose with over 90% efficiency when the carbodi-imide condensation method was used, GABA-T would not bind to the immobilized ligand in either a column or a stirred slurry, even though, in solution, it is a potent irreversible inhibitor (Fig. 1). This may indicate that both the carboxyl and amino groups of gabaculine, and hence of GABA, are essential for binding or, alternatively, that the enzyme and inhibitor cannot attain the correct orientation for binding once the inhibitor is partially immobilized.

An alternative approach was to purify GABA-T from a source that would yield milligram quantities and to raise antibody to this protein, to use as affinity ligands to purify the enzyme from locust. GABA-T was purified from sheep brain by the method of John & Fowler [5]. The pure enzyme had a specific activity of 4.10 μ mol/min per mg of protein and an M_r of $\sim$ 97000. From these values, a catalytic-centre activity (no. of molecules of substrate transformed/s per catalytic centre) was calculated to be 6.6 s⁻¹.

IgG from the serum of a rabbit inoculated with GABA-T from sheep brain was purified by the method

Fig. 1. Inhibition of GABA-T from locust by gabaculine

GABA-T (5 μg) was incubated in assay buffer at 25 °C for the indicated times, when the remainder of the assay constituents were added. The graph shows a family of inactivation curves illustrating time-dependent inhibition, and the inset shows the secondary plot of 1/K_{obs} versus 1/[I], from which a K_i (dissociation constant of the inhibitor from the E-I complex) of 3.2 μM and a K_{inact} of 0.18/min were determined. Gabaculine concentrations ([I]) were: Δ, none; ■, 0.5 μM; □, 1.0 μM; ○, 2.5 μM; ●, 5.0 μM.

Fig. 2. SDS/10%-polyacrylamide gel showing subunit size of GABA-T from locust supraoesophageal ganglia and sheep brain

Track 1: markers and their M_r values: from top to bottom, ovotransferrin (78 000), bovine serum albumin (66 250), ovalbumin (45 000), carbonic anhydrase (30 000), myoglobin (17 200) and cytochrome c (12 300). Track 2: GABA-T from locust. Track 3: GABA-T from sheep brain.

of Wright & Hunter [11]. Ouchterlony diffusion plates containing various dilutions of IgG were run against the sheep antigen (1 mg/ml) and extracts of lysed locust mitochondria concentrated by ultrafiltration. In addition, a solution (1 mg/ml) of GABASE (Sigma) containing GABA-T from *Pseudomonas fluorescens* was challenged with the sheep antibody. A positive precipitin reaction was detected with the sheep antigen only. The much more sensitive dot-immunobinding assay gave a positive result with the sheep antigen at antibody

dilutions in the range 1:50–1:800, but no reaction was observed with the locust antigen. A competitive enzyme-linked immunosorbent assay also failed to detect any cross-reactivity between the sheep antibody and the locust antigen at antibody dilutions in the range 1:50–

Table 1. Purification of GABA-T from locust head

1 unit of activity is expressed as the amount of enzyme catalysing the reduction of 1 μmol of NADP⁺/min under assay conditions; N.D., not determined.

	Volume (ml)	Protein (mg/ml)	Activity (units/ml)	Specific activity (units/mg of protein)	Yield (%)
Homogenate	700	6.0	0.016	0.002	100
Supernatant after 55 °C and pH 5.4	590	1.33	0.022	0.016	116
45–65 %-satd. (NH ₄) ₂ SO ₄	34	N.D.	0.24	N.D.	73
DEAE-cellulose effluent	51	1.55	0.12	0.081	54
Phenyl-agarose	50	0.17	0.09	0.53	40
Chromatofocusing	3.2	0.07	0.30	4.36	9

Fig. 3. Localization of enzyme activity on a discontinuous Percoll gradient

Fractionation on a discontinuous gradient (13.5/28/55% Percoll) of an enriched mitochondrial pellet, extracted from locust supraoesophageal ganglia as described in the text. Enzymes: ○, GABA-T; ●, succinate dehydrogenase. Recoveries were: GABA-T 90%, succinate dehydrogenase 88%.

1:500 in the presence and absence of normal goat serum used to decrease non-specific binding.

GABA-T from locust was finally purified by using the initial steps of the method of John & Fowler [5], followed by hydrophobic interaction chromatography and chromatofocusing; the purification is summarized in Table 1.

GABA-T from both sources has a similar M_r of ~97000, and is composed of similar-sized subunits

(Fig. 2). Both these values are slightly smaller than published values for other mammalian sources of the enzyme [12] and may be the result of proteolytic cleavage during purification [13].

Chromatofocusing of both enzymes over the narrow pH range of 7.0–5.0 yielded pI values of 6.7 and 5.5 for the locust and sheep GABA-T respectively.

The localization of GABA-T from locust supraoesophageal ganglia is confined to the mitochondrion; the profile of activity from a subcellular-localization study corresponds exactly to that of succinate dehydrogenase (Fig. 3), the mitochondrial marker, and is in agreement with the data from rat brain [14].

The purified enzyme from sheep (1 mg/ml) exhibits a decrease in A_{413} and an increase in A_{330} in the presence of 5 mM-GABA, which is not reversed after prolonged dialysis, but only after addition of excess 2-oxoglutarate. This spectral shift is indicative of a switch from the aldimine to ketimine form of the enzyme and signifies the presence of pyridoxal phosphate [15]. Not enough locust GABA-T was purified to allow the spectral determination of pyridoxal phosphate; however, as both enzymes are inhibited by gabaculine, it is probable that pyridoxal phosphate is present at the active site of GABA-T from the locust.

Table 2 summarizes the results of inhibition studies on both enzymes, by using substrate analogues and ligands known to interact with the mammalian GABA receptor and GABA-uptake system. Apart from muscimol, which is known to bind to both the mammalian and invertebrate GABA receptors and has been shown to be a competitive inhibitor of GABA-T from rabbit [16], gabaculine was the only compound to inhibit both enzymes. The mode of inhibition was time-dependent and irreversible. Isoguvacine and 3-aminopropane-1-sulphonic acid, both GABA agonists, and nipecotic acid, a GABA-uptake inhibitor, had no effect as GABA-T inhibitors. However, 2,4-diaminobutyric acid, a GABA-uptake inhibitor, was a weak competitive inhibitor of GABA-T from the locust, while baclofen, the GABA B-site agonist, was ineffective with both enzymes. The apparent ineffectiveness of receptor and uptake-site agonists and antagonists may be explained by the lack of a free amino group in those analogues structurally similar to GABA, i.e. 3-piperidine-carboxylic acid (nipecotic acid) and isoguvacine. Baclofen, although having the GABA-backbone free

Table 2. Effects of substrate analogues, GABA-receptor agonists and antagonists, and GABA-uptake inhibitors on GABA-T from sheep brain and locust ganglia

Abbreviations: N.E., no effect below 10 mM; N.D., not determined.

Inhibition	Enzyme . . .	Type of inhibition and K_i value	
		Locust	Sheep
Muscimol		Competitive, 2 mM	Competitive, 4 mM
Gabaculine		Irreversible, 3.2 μ M	Irreversible, 5.0 μ M
		Time-dependent	Time-dependent
Isoguvacine		N.E.	N.E.
Baclofen		N.E.	N.E.
Nipecotic acid		N.E.	N.E.
3-Aminopropane-1-sulphonic acid		N.E.	N.E.
DL-2,4-Diaminobutyric acid		Competitive, 50 mM	N.D.

amino group, is probably sterically inhibited by its aromatic nucleus, although Buu & Van Gelder [17] demonstrated that the dissociation constants for 3-phenyl-GABA and GABA were similar, thus implying that steric constraints at position 3 may not be critical to the absence of inhibition. With 3-aminopropane-1-sulphonic acid, the different negative-charge distribution of the sulphate group may be sufficient to prohibit binding or catalysis.

These initial results suggest that both enzymes are similar in many respects, except for their pI values and antigenicity. The Michaelis constants for GABA and 2-oxoglutarate are similar for both enzymes: 0.79 ± 0.16 mM and 0.27 ± 0.08 mM for GABA-T from locust, 2.2 ± 0.24 mM and 0.22 ± 0.11 mM for GABA-T from sheep. By comparison, the values for mouse brain enzyme are 1.1 mM and 0.25 mM for GABA and 2-oxoglutarate respectively [12], and those for rabbit brain are 18 mM and 5 mM [5]. The difference in pI may be responsible for the different behaviour of the enzymes in the purification protocols. Interestingly, the pI of GABA-T from the locust corresponds closely to the value of 6.8 for the human brain enzyme [18], and the pI for GABA-T from sheep brain is much closer to the value of 5.6 for the rat enzyme [19]. Both values, however, are completely different from that of 10.5 for GABA-T from the worm *Nippostrongylus brasiliensis* [19].

The fact that the two enzymes appear to be immunologically distinct is not entirely unexpected, since antibodies raised to rat L-glutamate 1-carboxy-lyase (EC 4.1.1.15), the enzyme which synthesizes GABA, gave very low cross-reactivity with glutamate carboxy-lyase from locust [20].

Further work is needed to map the binding sites of these proteins, by using known modification reagents in addition to specifically synthesized GABA analogues. This may determine whether subtle differences exist between the mammalian and insect proteins at the binding-site level, which could be exploited in the design of selective pesticides.

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