Competitive Inhibition of Mouse Brain γ-Aminobutyrate Aminotransferase by ATP

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Abstract. **The nucleotide ATP was shown to be a reversible inhibitor of partially purified γ-aminobutyrate aminotransferase isolated from mouse brain. This inhibition was of the competitive type with respect to the substrate, γ-aminobutyric acid (K_i = 3.7 ± 0.6 m*M*), but was noncompetitive with respect to both the second substrate α-ketoglutarate and the cofactor pyridoxal 5′-phosphate. Two analogues of ATP, ADP and GTP, also gave rise to an inhibition γ-aminobutyrate aminotransferase that was similar to that produced by ATP. These results are consistent with the view that mouse brain γ-aminobutyric acid aminotransferase could be under regulatory control by ATP and certain other nucleotides within the mitochondria.** [P.S.E.B.M. 1989, Vol 192]

γ-Aminobutyric acid (GABA) is widely recognized as the primary inhibitory transmitter in the mammalian central nervous system. Upon release from certain nerve terminals, the amino acid activates specific $GABA_A$ receptors on the postsynaptic membrane (1), engendering the opening of Cl^- channels in the membrane. The subsequent hyperpolarization of the postsynaptic nerve cell is the basis of GABA's inhibitory action (2).

The cessation of GABA neurotransmission occurs with the rapid removal of the transmitter from the synaptic gap by means of a high affinity, Na^{++}- and Cl^--dependent GABA pump (3, 4). Once taken up, GABA migrates to the mitochondria in preparation for its metabolic degradation by the action of an enzyme complex situated in the matrix (5). This macromolecular unit consists of GABA aminotransferase (GABA-T; EC 2.6.1.19) and succinic semialdehyde dehydrogenase (SSADH; EC 1.2.1.24) acting in unison to convert GABA to succinate via the intermediate succinic semialdehyde. The intermediate has little chance to accumulate since the activity of the dehydrogenase is in excess of that of GABA-T (6, 7).

Although L-glutamate decarboxylase is considered to be the rate-limiting step in GABA metabolism (8), inhibition of GABA-T *in situ* can lead to the elevation of GABA concentrations in the brain and to various physiologic and behavioral effects. For example, inhibition of this enzyme can confer an anticonvulsant action (9) and, in some instances, can lower blood pressure (10–12). In principle, the activity of GABA-T within the cell could be under some form of regulatory control (13). We now report that ATP can behave as a reversible inhibitor of mouse brain GABA-T *in vitro* and consequently can be considered as a candidate for a role in the regulation of the enzyme and thus GABA concentrations within the brain. A preliminary report of these findings has been published elsewhere (14).

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Materials and Methods

Animals. Adult male ICR mice were obtained from Harlan Sprague-Dawley, Indianapolis, IN, and kept in our animal facilities for at least 2 weeks before use.

Chemicals. All biochemicals were purchased from Sigma Chemical, St. Louis, MO. The nucleotides were in the form of sodium salts. [^{3}H]Aminobutyric acid (58 Ci/mmol) was obtained from Amersham, Arlington Heights, IL.

Purification of the Enzyme. Whole brains from 20 mice were pooled and homogenized in 9 volumes of ice-cold 0.32 *M* sucrose using a Potter-Elvehjem tissue grinder. The mixture was centrifuged at 1500*g* for 15 min. This and all centrifugations were carried out at 4°C. The supernatant was recentrifuged at 17,000*g* for 45 min. The resulting crude mitochondrial fraction was subjected to hypotonic shock by suspending in ice-cold water and stirring for 15 min. The mixture was centrifuged for 60 min at 100,000*g*. The pellet was suspended in 20 volumes of 10 m*M* sodium

phosphate buffer (pH 7.2) containing 0.1 m*M* pyridoxal 5′-phosphate, 1 m*M* EDTA, 0.5 m*M* dithiothreitol, and 0.5% Triton X-100. A Polytron was used to obtain a homogeneous mixture.

Solid ammonium sulfate was added to produce a 40% saturation. After centrifugation, the resulting pellet was discarded. More ammonium sulfate was then added to produce 70% saturation. Further centrifugation produced a pellet that was dissolved in the phosphate buffer and applied to a Sephadex G-200 column. The enzyme was eluted using the same phosphate buffer and 2-ml fractions were collected. Those fractions containing GABA-T activity were pooled and the enzyme was precipitated by the addition of ammonium sulfate (70% saturation). The precipitate was dissolved in buffer and, after dialysis overnight against 3 liters of buffer, was the source of the enzyme. This produced a fraction with a specific activity of about 2 μmol/minμmg protein, representing a 210-fold purification of the transaminase and a yield of 17%.

Enzyme Assay. The procedure of Sterri and Fonnum (15) was followed. This is a coupled assay in which [^{3}H]GABA is converted to [^{3}H]succinate by the combined action of GABA-T and SSADH in 50 m*M* Tris-HCl buffer (pH 7.5) containing 0.5 m*M* dithiothreitol, 3 m*M* NAD^+, and the appropriate amounts of GABA, α-ketoglutarate, and pyridoxal 5′-phosphate. All incubations were carried out at 37°C for 30 min unless otherwise stated. The labeled succinate was extracted with tri-*n*-octylammonium phosphate dissolved in chloroform. After the addition of toluene containing PPO and POPOP, the radioactivity was monitored in a Beckman LS 100 scintillation counter. Since this is a coupled assay, we felt it was essential to demonstrate that under our conditions the assay produced initial rates. Therefore, we measured enzyme activity as described above but over different periods of time up to a maximum of 60 min. For this experiment GABA, α-ketoglutarate, and pyridoxal 5′-phosphate concentrations were 1.5 m*M*, 1.0 m*M*, and 0.1 m*M*, respectively. We obtained a linear relationship between activity and time of incubation up to 45 min (data not shown). As a result 30 min was chosen for the standard assay condition.

For one experiment GABA-T activity was measured according to the method of Waksman and Roberts (16). This procedure depends on the direct assay of [^{3}H]glutamate formation from [^{3}H]α-ketoglutarate and does not rely on the presence of SSADH. The amino acid is separated from the keto acid by a Dowex 50-X8 cation exchange column. Incubations were performed for 30 min at substrate concentrations of 1 m*M* and a cofactor concentration of 0.1 m*M*. The reaction was terminated by the addition of 10% trichloroacetic acid and, after centrifugation, the supernatant was applied to the resin column. The eluate was collected and its radioactivity counted.

Protein Determination. The method of Lowry *et al.* (17) was used and standards were prepared from bovine serum albumin.

Results

Preliminary Experiments. Initially GABA-T activity was measured in the presence of 1 m*M* ATP at a fixed concentration of GABA, α-ketoglutarate, and pyridoxal 5′-phosphate (1.5 m*M*, 1 m*M*, and 0.1 m*M*, respectively). For these preliminary experiments, the source of the enzyme was the initial brain homogenate in 0.32 *M* sucrose. The reaction rate was inhibited by 46 ± 5.1%. To establish whether this effect was reversible or not, an aliquot of enzyme in 5 ml of 50 m*M* Tris-HCl buffer containing 0.5 m*M* dithiothreitol was incubated at 25°C with 1 m*M* ATP for various lengths of time from 1 to 45 min. After this exposure to ATP, the solution was diluted 100 times with buffer and assayed for GABA-T activity. No observable change in activity occurred when compared with that of identically treated enzyme which was not exposed to ATP (Table I). Furthermore, in another series of experiments the enzyme was diluted 100-fold before addition of ATP. However, on this occasion all ingredients for the assay were present during the preincubation with ATP except for GABA. After the appropriate period of time, [^{3}H]GABA was added and the enzyme activity monitored. As can be seen from Table I, we observed a remarkably constant degree of inhibition, indicating that time of exposure to ATP immediately before the enzyme assay was unimportant. These results strongly suggest that the inhibition of the enzyme by ATP is reversible.

To demonstrate that the effect of the nucleotides was on GABA-T rather than on SSADH, an experiment was carried following the procedure described by Waksman and Roberts (16). In the presence of 2 m*M* ATP, the enzyme was inhibited by 45 ± 6.4% during a 30-min incubation at 37°C (data not shown).

Kinetics of ATP Inhibition. The assay of GABA-T, using the purified enzyme, was carried out at various GABA concentrations but those of α-ketoglutarate and pyridoxal 5′-phosphate were fixed at 1 m*M* and 0.1 m*M*, respectively. The effects of 2 m*M* and 4 m*M* ATP were studied and plotted according to the method of Lineweaver and Burk (18). Each graph shown repre-

Table I. Reversibility of ATP Effects

Time of exposure to 1 m*M* ATP before assay (min)	% Inhibition	
	Without enzyme dilution	With 100-fold dilution
1	46 ± 5.1[a]	0
15	51 ± 7.8	0
30	40 ± 3.6	0
45	44 ± 8.5	0

[a] The values represent the mean (±SE) of three determinations.

sents data from one experiment, and each point was carried out in triplicate with variability not exceeding 15%. From the data in Figure 1, the K_m of the enzyme with respect to GABA was calculated as 0.51 m*M*. In the presence of 2 m*M* ATP, however, this value was increased to 1.1 m*M*, suggesting that the affinity of the enzyme for its substrate had decreased. In contrast, the V_{max} of the reaction was unaffected by ATP. By replotting the data after the method of Dixon (19), we obtained a K_i of 3.7 ± 0.6 m*M* as the mean of four experiments (graph not shown).

A similar series of experiments were performed except that the GABA concentration was held at 1.5 m*M* whereas that of α-ketoglutarate was varied. The concentration of cofactor was 0.1 m*M*. One of the graphs obtained is shown in Figure 2. The presence of 2 m*M* ATP did not alter the K_m and thus did not affect the affinity between the enzyme and its substrate. On the other hand, the V_{max} of the reaction was markedly reduced in proportion to the amount of inhibitor present.

A third experiment was performed in which both substrate concentrations were held constant at 1 m*M* but in which the pyridoxal 5′-phosphate concentration was varied (Fig. 3). As in the case of the previous experiment, the V_{max} was reduced by ATP. However, although the K_m was unchanged by the presence of 5 m*M* ATP, it was increased in the presence of 10 m*M* ATP. This indicates that the inhibitor did not interact with the cofactor binding site on the enzyme at the lower ATP concentration but appeared to increase the affinity between the enzyme and pyridoxal 5′-phosphate at the higher concentration. We obtained a K_i of 8.7 ± 1.2 mM as the mean of four experiments.

Experiments similar to those described above were performed using ADP and GTP instead of ATP. As in the case of the latter inhibitor, these nucleotides appeared to inhibit GABA-T in a competitive manner

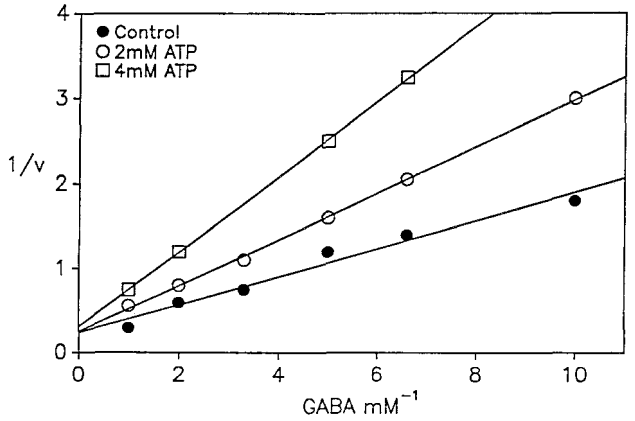

Figure 1. Double reciprocal plot of the effects of ATP on initial rates of mouse brain GABA-T with varying GABA concentrations. α-Ketoglutarate and pyridoxal 5′-phosphate concentrations were 1 m*M* and 0.1 m*M*, respectively. Assay carried out in 50 m*M* Tris-HCl (pH 7.5). Velocity (v) = μmol/min/mg protein.

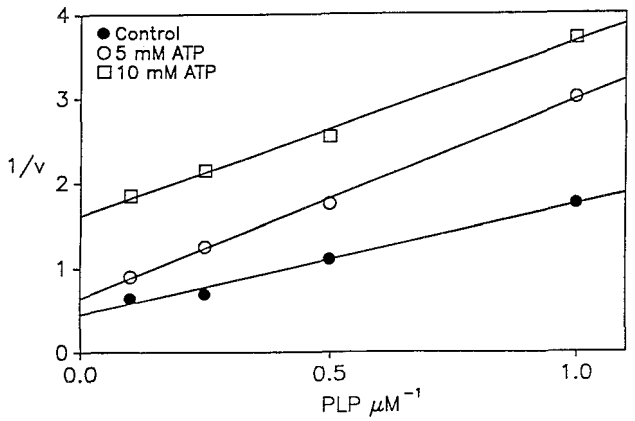

Figure 3. Double reciprocal plot of the effects of ATP on initial rates of GABA-T with varying pyridoxal 5′-phosphate (PLP) concentration. GABA and α-ketoglutarate concentrations were both 1 m*M*. Assay was carried out in 50 m*M* Tris-HCl (pH 7.5). Velocity (v) = μmol/min/mg protein.

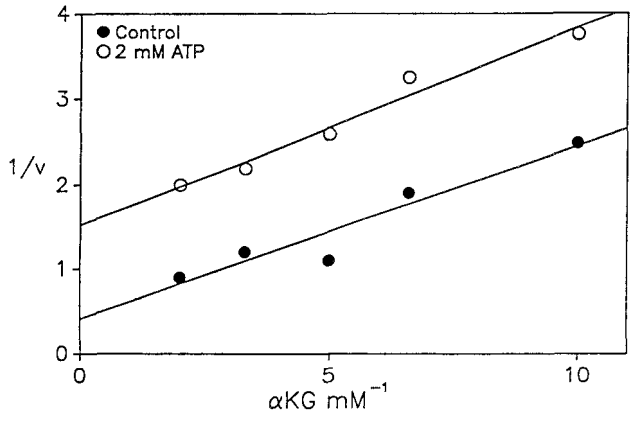

Figure 2. Double reciprocal plot of the effects of ATP on the initial rates of GABA-T with varying α-ketoglutarate (α-KG) concentrations. GABA and pyridoxal 5′-phosphate concentrations were held constant at 1 m*M* and 0.1 m*M*, respectively. Assay performed in 50 m*M* Tris-HCl (pH 7.5). Velocity (v) = μmol/min/mg protein.

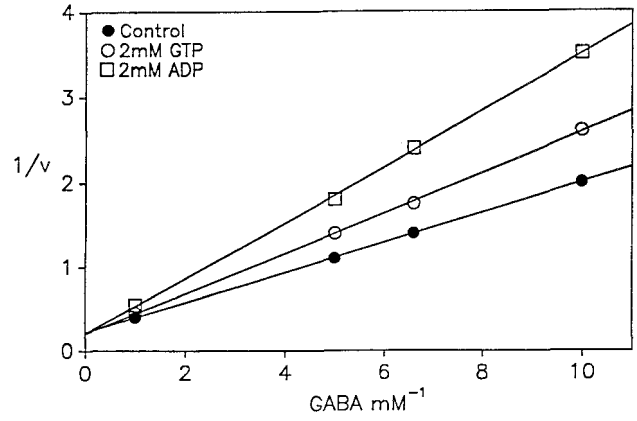

Figure 4. Double reciprocal plot of the effects of ADP and GTP on the initial rates of GABA-T with respect to varying GABA concentrations. α-Ketoglutarate and pyridoxal 5′-phosphate concentrations were 1 m*M* and 0.1 m*M*, respectively. Assay performed in 50 m*M* Tris-HCl (pH 7.5). Velocity (v) = μmol/min/mg protein.

Table II. Kinetic Analysis of Nucleotide Inhibition of GABA-T

Substrate/ cofactor	Nucleotide	Mode of inhibition	K_i (mM)
GABA	ATP	Competitive	3.7 ± 0.6[a]
	ADP	Competitive	2.7 ± 1.2
	GTP	Competitive	6.2 ± 1.7
α-KG	ATP	Noncompetitive	27.0 ± 9.9
	ADP	Noncompetitive	>100
	GTP	Noncompetitive	34.0 ± 15.5
PLP	ATP	Noncompetitive	8.7 ± 1.2
	ADP	Noncompetitive	17.5 ± 8.9
	GTP	Noncompetitive	54.0 ± 12

[a] The values represent the mean (±SE) of three experiments.

with respect to GABA (Fig. 4). On the other hand, they exhibited noncompetitive inhibition with respect to both α-ketoglutarate and pyridoxal 5′-phosphate (data not shown). A summary of the type of inhibition and K_i of each inhibitor are shown in Table II.

Discussion

These experiments were initiated to ascertain whether ATP was capable of influencing the kinetics of mouse brain GABA aminotransferase. Recently Carr *et al.* (20) published evidence that GABA-T isolated from pig brain could be phosphorylated under specific conditions in the presence of ATP. This phosphorylation, however, did not alter the kinetics of the enzyme. Because of our continuing studies of GABA-T from mouse brain we were interested to know what effect ATP would have on this enzyme. We were intrigued to observe a reduction of activity when we assayed a portion of brain homogenate in the presence of 1 mM ATP. We assumed, in light of the report from Carr *et al.* (20), that we had managed to phosphorylate the enzyme and that this led to the observed reduction in activity. Consequently, it came as a surprise to observe that this inhibition was reversible. Our results clearly demonstrate that mouse brain GABA-T is inhibited by ATP but there is no evidence that these effects are mediated by a phosphorylation of the enzyme.

The present results provide evidence that ATP (and indeed ADP and GTP) reduces the affinity between GABA-T and its substrate GABA. One interpretation is that these nucleotides are competitive inhibitors. However, if their structures are compared with that of GABA it is not obvious they are structural analogues of this substrate—surely a prerequisite for competitive inhibition? If these inhibitors are not competing with GABA for the active site of the enzyme, perhaps they bind to another site on the enzyme and act as allosteric effectors. By so doing, these compounds could alter the conformation of the protein and affect the binding of substrate in such a way that it appears that competitive inhibition is occurring.

Whether or not these observations have any physiologic significance is not known. It has long been accepted that in GABA metabolism the rate-limiting step is the formation of this neurotransmitter from glutamate (8). Because of this there is less likelihood that GABA-T is under any regulatory control in the intact brain. This having been said, we know that the *in vivo* inhibition of the enzyme can have physiologic and behavioral consequences. For example, the administration of certain drugs that can inhibit GABA-T has been reported to be associated with a reduction in mean arterial pressure (10–12) and to be implicated in anticonvulsant action (9). Thus, it is known that alterations in GABA-T activity *in vivo* have marked consequences. It is conceivable that in the mitochondria the concentrations of various nucleotides exert some influence over the catalytic activity of GABA-T and as a result participate in the regulation of GABA metabolism. The levels of ATP and ADP in the vicinity of GABA-T are of the order of 10^{-3} M (21) and consequently these nucleotides would be in an ideal position to act as regulators of GABA-T activity, if they play such a role. It it transpires that this is indeed the case, then GABA-T would be the second enzyme involved in GABA metabolism to come under the influence of ATP since there is considerable evidence that L-glutamate decarboxylase, too, can be regulated by this nucleotide (8).

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