Interactions between 4-Aminobutyrate Aminotransferase and Succinic Semialdehyde Dehydrogenase, Two Mitochondrial Enzymes*

(Received for publication, May 7, 1984)

William G. Hearl and Jorge E. Churchich
From the Department of Biochemistry, University of Tennessee, Knoxville, Tennessee 37996-0840

Physical interactions between the enzymes involved in the catabolism of the neurotransmitter 4-aminobutyrate were detected by means of affinity chromatography and fluorescence techniques.

By immobilizing one enzyme (4-aminobutyrate aminotransferase) indirectly through antibodies bound to protein A-Sepharose, it was possible to demonstrate that succinic semialdehyde dehydrogenase interacts with the aminotransferase at neutral pH and ionic strength values higher than 0.2.

Increasing the ionic strength of the medium results in dissociation of the "enzyme cluster."

Binding of succinic semialdehyde dehydrogenase to the aminotransferase tagged with a fluorescent probe was detected by polarization of fluorescence measurements at neutral pH. Upon saturation of the aminotransferase with succinic semialdehyde dehydrogenase, the polarization of fluorescence increases from 0.13 to 0.21.

The results are consistent with a model in which one molecule of succinic semialdehyde dehydrogenase is bound to one molecule of 4-aminobutyrate aminotransferase with an equilibrium dissociation constant of 0.1 μM. Since the concentrations of both enzymes in the mitochondrial matrix have been estimated to be around 2 μM, the results obtained with the purified mitochondrial enzymes strongly suggest that the aminotransferase is saturated with succinic semialdehyde dehydrogenase to form a stable enzymatic complex under in vivo conditions.

4-Aminobutyrate aminotransferase together with succinic semialdehyde dehydrogenase and glutamate decarboxylase are the main components of the 4-aminobutyrate shunt. They participate in the metabolism of the neurotransmitter 4-aminobutyrate.

The aminotransferase, isolated from brain tissues, is a dimer consisting of subunits of 50,000 molecular weight each (1). Succinic semialdehyde dehydrogenase, on the other hand, contains four identical subunits of 40,000 molecular weight each (2). The reaction catalyzed by the dehydrogenase proceeds in the direction of formation of succinic acid and NADH; and it is well established with the enzyme is sensitive to inhibition by the substrate succinic semialdehyde as well as by the product NADH (2).

Accumulation of succinic semialdehyde, a compound highly toxic to the cell, is prevented by 4-aminobutyrate aminotransferase which catalyzes the reversible reaction between succinic semialdehyde and glutamate. Since both enzymes are in the mitochondrial matrix participating in successive reactions in the same metabolic pathway, it seems reasonable to consider the possibility that they form part of a multienzyme complex. Such "enzyme clusters" could provide a suitable device in the control of metabolic cross-roads by increasing the efficiency of the sequential catalytic steps through the decrease of transit time for the passage of metabolites between the enzymes. In addition, "enzyme clusters" could prevent the leakage of intermediate metabolites which are harmful to the cell, i.e. succinic semialdehyde.

It is the purpose of this research to investigate the interaction between 4-aminobutyrate aminotransferase and succinic semialdehyde dehydrogenase and to define the experimental conditions under which such interactions take place.

EXPERIMENTAL PROCEDURES

Purification of Enzymes—4-Aminobutyrate aminotransferase was purified from pig brain as described by Churchich and Moses (1). This preparation has a specific activity of 20 units/mg at 25 °C.

Succinic semialdehyde dehydrogenase was prepared as previously described (3). The preparation had a specific activity of 5 units/mg at 25 °C. Both enzymes migrated as a single band on polyacrylamide gel electrophoresis. One unit of enzyme activity is defined as the amount of enzyme which will produce 1 μmol of NADH per min at 25 °C.

Protein concentrations were determined using the Bio-Rad protein assay (Bio-Rad) using bovine serum albumin as a standard.

Enzymatic Assays—Enzymes in solution were assayed as previously described (4). 4-Aminobutyrate aminotransferase was assayed by the coupled assay method using succinic semialdehyde dehydrogenase as the second enzyme. Activity was measured by monitoring the change in absorbance at 340 nm for at least 2 min. Succinic semialdehyde dehydrogenase was assayed directly by observing the increase of absorbance at 340 nm due to reduction of NADH (2).

Imobilized enzyme 4-aminobutyrate aminotransferase was assayed by running the substrate mixture consisting of 30 mM 4-aminobutyrate, 10 mM 2-oxoglutarate in 0.1 M potassium pyrophosphate (pH 8.4) through the column with a peristaltic pump at a flow rate of 0.7 ml/min, and aliquots (0.5 ml) were assayed for succinic semialdehyde content using the fluorometric method previously described (4).

Preparation of PLP-Sepharose—PLP-Sepharose was prepared as described by Ikeda and Fukui (5) with a few modifications. The starting material was aminohexyl Sepharose rather than Sepharose 4B, and the coupling reaction was allowed to proceed for 18 h. Derivatized PLP-Sepharose was stored in 50% glycerol at -20 °C and contained approximately 10 μmol of PLP/ml of gel. Columns were routinely packed in a disposable plastic syringe with a total bed volume of 0.1 ml. For enzymatic assays, 100 μg of the aminotransferase were applied (>99% retained), and a single column was used for a full set of kinetic assays. The column was saturated with the aminotransferase (300 μg/ml column) and subsequently reduced with NaBH₄, prior to use as an affinity column.

The abbreviations used are: PLP, pyridoxal 5-phosphate; PBS, 50 mM potassium phosphate (pH 7.4) containing 0.15 M NaCl; IgG, immunoglobulin G.

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Preparation of Antiserum—A rabbit monoclonal antiserum against porcine 4-aminobutyrate aminotransferase was prepared as described by Isemura et al. (6). A New Zealand White rabbit received three subcutaneous injections at 2-week intervals of an emulsified mixture (1.5 ml) of 4-aminobutyrate aminotransferase (1 mg/ml) and Freund's complete adjuvant. Blood was drawn from the ear vein 3 weeks after the final injection and thereafter. The presence of anti-aminotransferase antibody was detected by immunodiffusion using the method of Ochterlony and Nilsson (7).

Purification of Immunoglobulin G—Serum collected from an immunized rabbit was adjusted to 40% saturation in ammonium sulfate. The precipitate was dissolved in 50 mM potassium phosphate containing 0.15 M NaCl (PBS) at pH 7.4. The ammonium sulfate precipitation was repeated two times and the final precipitate dialyzed against PBS. The dialysate (250 mg/ml) was loaded onto a protein A-Sepharose column (5-ml bed volume) equilibrated in PBS, and the column was washed with 10 bed volumes of the starting buffer. The IgG fraction was eluted with 0.1 M glycine/HC1 buffer, pH 2.8, directly into tubes containing enough 1 M potassium phosphate (pH 7.6) to bring the pH to 7.0 and then dialyzed against PBS. The final IgG preparation was stored at -20°C prior to use.

Western Blots—Protein was transferred from polyacrylamide gels to nitrocellulose filters electrophoretically using a modification of the procedure of Bittner et al. (8). The nitrocellulose bound protein was probed with anti-aminotransferaseantiserum, and specific antibody-antigen interactions were detected using 125I-labeled goat anti-rabbit IgG according to the procedure of Renart et al. (9).

Preparation of Modified 4-Aminobutyrate Aminotransferase—Iodoacetamide fluorescein-labeled aminotransferase was prepared as described by Kim and Churchich (10). The degree of labeling was determined spectrophotometrically using an extinction coefficient of 3.4 x 104 M-1 cm-1 at 490 nm. The incorporation of 0.92 mol of dye/mol of enzyme was observed.

Reduced 4-aminobutyrate aminotransferase was prepared by incubation of the enzyme with 10 mM ML P for 60 min at 4°C and then treating with excess NaBH4 for 30 min at 0°C. Excess of free reagent was removed by gel filtration through Sephadex G-25 equilibrated with 10 mM potassium phosphate (pH 7.2) containing 0.1 M mercaptooctanol.

Spectroscopy—Polarization of fluorescence measurements was performed on a SLM 4800 spectrofluorimeter. The excitation set was at 440 nm, and the fluorescent polarized light emitted by the sample was passed through a Corning Glass filter C.S. 3-69.

UV and visible measurements were performed in a Cary model 15 spectrophotometer. Fluorescence measurements were recorded on a spectrofluorimeter equipped with two Haush and Lomb monochromators.

Polyacrylamide Gel Electrophoresis—Polyacrylamide gel electrophoresis was performed according to the procedure of Davis (11). Discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out at 25°C as described by Laemmli (12). The gel (9% acrylamide) contained 0.1% sodium dodecyl sulfate.

Protein bands were detected by staining with Coomassie blue dye for 1 h and subsequently destained in a solution containing 5% methanol, 7.5% acetic acid in water.

Materials—Pig brains were obtained from Lays Meat Packing Company. Aminohexyl Sepharose, protein A-Sepharose, Sephadex G-25, and Sephacryl S-300 were obtained from Pharmacia. Pyridoxal-5-phosphate and sodium borohydride were purchased from Sigma. Iodoacetamide fluorescein was from Molecular Probes. Protein standards are from Sigma. The 125I-labeled goat antirabbit IgG was a generous gift from Dr. John Koontz and the rabbit a gift from Dr. Jayant Joshi.

RESULTS

Affinity Chromatography—Native and resolved 4-aminobutyrate aminotransferase bind to pyridoxal-5-P derivatized Sepharose and remain firmly attached to the matrix as revealed by the absence of enzyme in fractions eluted from the Sepharose column by increasing concentrations of phosphate buffers at pH 7.

Enzymatically inactive 4-aminobutyrate aminotransferase, prepared by saturating the holoenzyme with excess pyridoxal-5-P followed by reduction with NaBH4, failed to bind to derivatized PLP-Sepharose. Since the catalytic sites of the inactive species are blocked by reduction of NaBH4, it appears that empty cofactor binding sites are needed for recognition of pyridoxal-5-P molecules coupled to the Sepharose matrix.

Imobilized 4-aminobutyrate aminotransferase is catalytically competent; the enzyme is stable over a period of 8 h at 25°C, and full retention of catalytic activity was observed in samples of immobilized enzyme stored at 4°C for 1 week. Immobilized 4-aminobutyrate aminotransferase exhibits maximum catalytic activity at pH 8.4, but the activity profile is narrower than that observed for free enzyme at 25°C (Fig. 1).

The kinetic parameters of immobilized enzymes are affected by factors such as diffusion and steric hindrance. The relative contribution of diffusion effects on the kinetic behavior of immobilized enzymes can be assessed from an Arrhenius plot (13). In the case of immobilized 4-aminobutyrate aminotransferase, the plots of log v versus 1/T yielded a straight line over the temperature range 4-40°C, indicating that diffusion effects do not play a significant role in the reaction catalyzed by the enzyme.

Although the effects of steric hindrance on the catalytic parameters of the immobilized enzyme were not assessed, the primary plots of 1/v versus 1/S yielded a set of parallel lines consistent with a binary complex mechanism for the reaction catalyzed by the aminotransferase. The Ks and Vmax values obtained from secondary plots are included in Table I. It is evident that the immobilized enzyme exhibits Ks values for the substrates which are higher than the corresponding values for free enzyme. This well behaved immobilized enzyme system was then used to detect specific interactions with succinic semialdehyde dehydrogenase.

To this end, a sample of succinic semialdehyde dehydrogenase (1 mg) was allowed to equilibrate with immobilized aminotransferase at 25°C for 15 min, and the PLP-Sepharose column (0.2 ml) eluted with phosphate buffers (pH 7.2) of increasing ionic strengths. Enzymatic assays of the effluent revealed full retention of succinic semialdehyde dehydrogenase at phosphate concentrations of 0.1 M at neutral pH.

In order to demonstrate that retention of succinic semialdehyde dehydrogenase is not due to binding of this enzyme to pyridoxal-5-P molecules covalently attached to the Seph-

![Fig. 1. Effect of immobilization on the pH activity profile of 4-aminobutyrate aminotransferase.](https://example.com/image)

<table>
<thead>
<tr>
<th>Table I</th>
<th>The kinetic parameters of immobilized aminotransferase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ks</td>
<td>Km</td>
</tr>
<tr>
<td>(mM)</td>
<td>(μmol·min⁻¹)</td>
</tr>
<tr>
<td>Free</td>
<td>0.1</td>
</tr>
<tr>
<td>Immobilized</td>
<td>2.0</td>
</tr>
</tbody>
</table>

* Kinetic constants for 2-oxoglutarate.

** Kinetic constants for 4-aminobutyrate.
when purified succinic semialdehyde dehydrogenase was applied to reduced immobilized aminotransferase, it was found that elution of succinic semialdehyde dehydrogenase from the column occurs at phosphate concentrations higher than 0.1 M as shown by the results included in Fig. 2.

Other proteins, i.e. bovine serum albumin, ovalbumin, and glutamate dehydrogenase, are not retained by immobilized aminotransferase when the concentration of eluting buffer is 0.01 M at pH 7.2.

These results lend strong support to the contention that retention of succinic semialdehyde aminotransferase by immobilized aminotransferase is not due to Schiff's base formation between pyridoxal-5-P coupled to Sepharose and the dehydrogenase.

Further support for specific interaction between 4-aminobutyrate aminotransferase and succinic semialdehyde dehydrogenase was derived from affinity chromatography experiments using aminotransferase-specific antibodies bound to protein A-Sepharose. Data obtained from Western blots probed with the anti-aminotransferase IgG showed that the antibody specifically recognizes the enzyme (data not shown).

It is known that binding of IgG molecules to protein A occurs via the Fc portion of the immunoglobulin, thus leaving the Fab antigen binding site readily available for interaction with antigen (14). The obvious advantage of such a system lies in the fact that this indirect and gentle method of immobilization excludes any physical modification of the enzyme as a result of its immobilization. Protein A-Sepharose loaded with purified anti-4-aminobutyrate aminotransferase was washed with increasing concentrations of saline buffer (I = 0.2) and then equilibrated with 10 mM phosphate buffer (pH 7.4).

Enzyme mixture (1 mg each aminotransferase and succinic semialdehyde dehydrogenase) was applied to a small column (2 x 1 cm) packed with anti-aminotransferase bound to protein A-Sepharose and eluted with a linear gradient of NaCl in a 10 mM phosphate buffer (pH 7.4). Fractions collected from the column were assayed for catalytic activity and used for determination of protein content. As shown in Fig. 3, succinic semialdehyde dehydrogenase is eluted at NaCl concentrations higher than 0.2 M, whereas 4-aminobutyrate aminotransferase remains firmly bound to the antibody under similar experimental conditions.

In control experiments protein A-Sepharose carrying anti-aminotransferase (4-aminobutyrate aminotransferase omitted) failed to retain succinic semialdehyde dehydrogenase at phosphate concentrations of 0.01 M (pH 7.4).

Thus, both experiments, i.e. affinity chromatography with 4-aminobutyrate aminotransferase immobilized directly and indirectly to a Sepharose matrix, reveal specific interactions of this enzyme with succinic semialdehyde dehydrogenase.

**Fig. 2.** The elution of succinic semialdehyde dehydrogenase from an aminotransferase-Sepharose column (0.5-ml bed volume). Reduced aminotransferase (400 µg) covalently attached to the Sepharose matrix was equilibrated with 10 mM potassium phosphate (pH 7.2) containing 1 mM 2-mercaptoethanol. 500 µg of succinic semialdehyde dehydrogenase were applied to the column and washed with 10 ml of 10 mM potassium phosphate buffer (pH 7.2). The column was eluted with equal volumes (5 ml) of increasing potassium phosphate buffer (pH 7.2). Fractions eluted from the column were monitored by absorbance measurements at 280 nm and assayed for succinic semialdehyde dehydrogenase activity.

**Fig. 3.** Elution of succinic semialdehyde dehydrogenase from anti-aminotransferase-protein A-Sepharose. A protein A-Sepharose column (2 ml) was loaded with purified antibody against 4-aminobutyrate aminotransferase and washed with phosphate buffer (pH 7.4) containing 0.2 mM NaCl. Then the column was equilibrated with 10 mM phosphate buffer (pH 7.4). A mixture of 4-aminobutyrate aminotransferase (1 mg) and succinic semialdehyde dehydrogenase (DHase) (1 mg) in 0.5 ml of phosphate buffer (pH 7.4) was applied to the column and eluted with 10 ml of the same buffer. A linear NaCl gradient made up of equal volumes (15 ml) of 0 and 2 mM NaCl in 10 mM phosphate buffer (pH 7.4) was applied to the column. Fractions collected from the column were assayed for succinic semialdehyde dehydrogenase activity.

**Fig. 4.** Gel filtration. A sample containing 1 mg of 4-aminobutyrate aminotransferase and 1 mg of succinic semialdehyde dehydrogenase was applied to a Sephacryl S-300 column (15 x 1 cm) equilibrated with 10 mM potassium phosphate (pH 7.2). The column was eluted with the same buffer, and fractions (0.4 ml) were assayed for succinic semialdehyde dehydrogenase (•) and 4-aminobutyrate aminotransferase (○) activities. The column was calibrated with the following standards: ferritin (440,000), human IgG (160,000), bovine serum albumin (68,000), and 4-aminobutyrate aminotransferase (GABA-T) (100,000). In the inset the elution volumes are plotted against the log of the molecular weight of the standard proteins.
The fraction of ligand bound

\[ \alpha = \frac{A}{L} \]  

is the ratio of bound fluorescein-aminotransferase \((PL)\) to the total concentration of free fluorescein-aminotransferase \((L_0)\).

It is related to the emission anisotropy values of free \((A_f)\) and bound \((A_b)\) fluorescein-aminotransferase by means of Equation 3.

\[ \alpha = \frac{(A - A_f)}{(A_f - A_b)} \]  

\( A \) is the emission anisotropy value observed when free and bound fluorescein-aminotransferase are in equilibrium. From a plot of \( \alpha \) versus concentration of succinic semialdehyde aminotransferase (Fig. 6), one can infer an apparent association constant of around 0.1 \( \mu M \) for a one to one complex formed as a result of binding of labeled aminotransferase to succinic semialdehyde dehydrogenase.

**DISCUSSION**

In the present studies we have demonstrated the existence of physical interactions between mitochondrial enzymes involved in the catabolism of the neurotransmitter 4-aminobutyrate.

Using directly immobilized 4-aminobutyrate aminotransferase in chromatographic studies, it was possible to show that the aminotransferase recognizes succinic semialdehyde dehydrogenase at neutral pH.

Interactions between the two enzymes were also detected by means of antibodies bound to protein A coupled to a Sepharose matrix. Instead of covalently linking the aminotransferase to pyridoxal-5-P Sepharose, the mitochondrial enzymes were immobilized indirectly via anti-aminotransferase. This indirect method of immobilization excludes any chemical modification of the aminotransferase brought about by its direct attachment to the Sepharose matrix.

When 4-aminobutyrate aminotransferase was immobilized via antibodies, it was found that about 1 mol of succinic semialdehyde dehydrogenase was bound per mol of immobilized aminotransferase at NaCl concentrations lower than 0.2 M. At increasing salt concentrations, the interaction between 4-aminobutyrate aminotransferase and succinic semialdehyde dehydrogenase was considerably reduced, indicating involvement of electrostatic forces in the recognition process.

Additional support for specific interactions between the two mitochondrial enzymes was derived from polarization of fluorescence measurements conducted on fluorescein-aminotransferase mixed with increasing concentrations of succinic semialdehyde dehydrogenase. The results of these experiments clearly established the formation of stable enzyme complexes characterized by a dissociation constant of approximately 0.1 \( \mu M \). Since the estimated concentration of 4-aminobutyrate aminotransferase and succinic semialdehyde dehydrogenase in mitochondria is approximately 2 \( \mu M \) each (16), the results obtained under in vitro conditions strongly suggest that the aminotransferase is saturated with succinic semialdehyde dehydrogenase under in vivo conditions.

The protein concentration in the mitochondrial matrix has been estimated to be about 500 mg/ml (17); and it is evident that high local concentrations of proteins exert several effects on the properties of the medium in which enzymes act.

Thus the medium becomes more viscous, and the rate of macromolecular diffusion may be reduced by an order of magnitude.

An average protein concentration of 500 mg/ml means that individual enzyme molecules will be in close proximity to one another, and this will tend to promote interaction between enzymes within the mitochondria matrix which would not occur in dilute solutions. Indeed, several publications have appeared lately indicating the existence of physical interactions between enzymes of the aspartate-malate shuttle and the citric acid cycle (18–21). The organization of the citric
acid cycle enzymes as a complex would facilitate the creation of a special microenvironment around the cycle; the cell would acquire the property of maintaining high flux of substrates through the cycle with a moderate number of intermediate molecules.

It is well established that the brain differs from other organs in having an alternative route of oxidation through α-ketoglutarate, i.e. 4-aminobutyrate bypass.

The reactions catalyzed by enzymes of the 4-aminobutyrate, i.e. glutamate decarboxylase, 4-aminobutyrate aminotransferase, and succinic semialdehyde dehydrogenase, lead to the formation of succinate, CO₂, and NADH.

\[ \alpha\text{-Ketoglutarate} + \text{NAD}^+ \rightarrow \text{succinate} + \text{CO}_2 + \text{NADH} + \text{H}^+ \]

Thus, the combined action of 3 enzymes allows succinate to re-enter the tricarboxylic acid cycle. The quantitative contribution of the 4-aminobutyrate bypass to glucose oxidation in the brain has been estimated to be 8–10% of the total flux through the trichloroacetic acid cycle (22).

In the case of 4-aminobutyrate aminotransferase and succinic semialdehyde dehydrogenase, two components of the 4-aminobutyrate shunt, it can be envisaged that close association between both enzymes will facilitate direct transfer of intermediates among the catalytic centers, thereby preventing release of free succinic semialdehyde which is completely converted to succinic acid.

Although the present studies have failed to detect any specific interaction between 4-aminobutyrate aminotransferase and glutamate dehydrogenase, it is conceivable that succinic semialdehyde dehydrogenase interacts with enzymes of the citric acid cycle, such as the flavoprotein succinate dehydrogenase, which catalyzes the dehydrogenation of succinate to fumarate.

Acknowledgements—It is a pleasure to acknowledge the continuous support of our colleagues Dr. John Koontz and Dr. J. Joshi, who have provided valuable advice and help in the immunological experiments.