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Effects of the Anticonvulsant Drugs on Succinic Semialdehyde Reductase from Bovine Brain

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Abstract—We have previously reported that an NADPH-dependent succinic semialdehyde reductase was purified homogeneously from bovine brain by several chromatographic procedures, and was found to be a monomeric protein with a molecular mass of 28 kDa (Cho *et al.*, Eur. J. Biochem. 1993). Since succinic semialdehyde is an important intermediate in the γ-aminobutyrate(GABA) shunt and GABA level is associated with various forms of human neurological disorders, we have investigated the effects of anticonvulsant drugs on the succinic semialdehyde reductase. Among the drugs tested, sodium valproate and diphenylhydantoin inhibited the enzyme activity, while some other drugs, barbiturate and chlorpromazine, had no inhibitory effects on the enzyme activity. The purified enzyme was also injected as an immunogen into Balb/c mice to obtain monoclonal antibodies (mAb) and several mAbs to the protein were produced from the fusion experiments.

Keywords □ γ-hydroxybutyrate, brain, succinic semialdehyde reductase, GABA shunt, monoclonal antibodies.

Succinic semialdehyde (SSA) is an intermediate of the γ-aminobutyrate (GABA) shunt pathway. SSA is formed by transamination of the major inhibitory neurotransmitter GABA by GABA transaminase (GABA-T) and the cerebral concentration of SSA is normally very low in contrast to GABA (Matsuda and Hoshino, 1977). It is well known that the abnormal levels of GABA in brain have been associated with a variety of neurological disorders including seizures, convulsion, epilepsy, Huntington's disease, and Parkinsonism (Perry *et al.*, 19973; Tower, 1970; Lloyd *et al.*, 1977). However, SSA can also be reduced to γ-hydroxybutyrate (GHB) in brain tissue (Fishbein and Bessman, 1964) and a number of oxidoreductases which catalyze the reduction of SSA to GHB have been identified as NADPH-dependent aldehyde reductases (Tabakoff and Von Wartburg, 1975; Cash *et al.*, 1979; Hearl and Churchich, 1985). GHB is a normal constituent of mammalian brain (Roth and Giarman, 1970; Roth, 1970) and known to play neurophysiological roles (Turner and Whittle, 1983). Systemically administered GHB has been shown to have a number of pharmacological effects. These include on anesthetic action and natural sleeping, and anesthetic doses of GHB have been shown to increase the dopamine level in brain (Gessa *et al.*, 1966; Godchalk *et al.*, 1977).

Despite many interesting observations, reduction of SSA to GHB has not received considerable attention, compared to that of GABA to SSA, since the mechanism by which this reductive pathway operates *in vitro* has not been clearly known yet. However, a specific binding site for GHB with high affinity has been detected in synaptic membrane preparations (Benavides *et al.*, 1982) and a fairly specific SSA reductase from brain tissues has been isolated. In addition, the studies of reduction of SSA to GHB using rat and pig brain as the enzyme source strongly support that GHB biosynthesis may be an important step in the GABA shunt of phamacological interest (Rivett et al., 1981; Hearl and Churchich, 1985).

In this work, we have isolated from bovine brain an NADPH-dependent SSA reductase which can interconvert SSA and GHB, and examined the inhibitory effects of some anticonvulsant drugs on the enzyme. We also produced monoclonal antibodies to the enzyme which can be used as specific probes in investigating its expression in brain tissues.

Materials and Methods

Materials

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NADPH, $NADP^+$, succinic semialdehyde, bovine serum albumin, γ-hydroxybutyrate, DL-glyceraldehyde, EDTA, and other chemicals were purchased from Sigma Chemical Co. Mono-Q, Superose-12, CM-Sepharose, and Blue-Sepharose were obtained from Phamacia/LKB, Ltd and hydroxyapatite was from Bio-Rad. Bovine brains were obtained from Majang Slaughterhouse, Seoul. Fetal bovine sera were obtained from Hyclone and polyethylene glycol 1500 from Sigma Chemical Co. All other chemicals, media, and antibiotics for the production of monoclonal antibodies were purchased from Gibco-BRL. Goat anti-mouse IgG antibodies were obtained from Jackson ImmunoResearch.

Purification and Enzymatic Assays of Succinic Semialdehyde Reductase

The succinic semialdehyde reductase from bovine brain was purified according to a procedure previously described (Cho *et al.*, 1993). The oxidation of NADPH to $NADP^+$ was measured by following the decrease in absorbance at 340 nm by the method of Hearl and Churchich (1985). All assays were performed in duplicate, and initial velocity data were correlated with a standard assay mixture containing succinic semialdehyde (120 μM) and NADPH (50 μM) in 0.1 M potassium phosphate, pH 7.2 at 25℃. One unit of enzyme activity was defined as the amount of enzyme required to oxidize 1 μmol of NADPH per min at 25℃. Initial velocity data were fitted by a least square method to the double reciprocal transformation of equation 1.

$$v = V_{max}\ [S]/(Km + [S]) \quad (1)$$

Protein concentration was estimated by the Bradford procedure with a bovine albumin standard (Bradford, 1976).

Production of monoclonal antibodies

The immunogen purified as described above was mixed with an equal volume of complete Freund's adjuvant by sonication and injected into female BALB/c mice (6~8 weeks od). The first injection was followed by three booster injections at 3- to 4- week intervals. The feeder layer cells were prepared one day before fusion.

The fusion experiments were performed as follows (Galfre and Milstein, 1981; Choi and Jeon, 1989). In brief, prepared spleen and SP2/o cell suspensions were combined and 1 m*l* of 50% polyethylene glycol 1500 in incomplete Dulbelco's modified Eagel's medium (DME) was added slowly. The fusion process was allowed to continue for 90 sec at 37℃ and stopped by adding incomplete DME. In order to avoid an osmotic shock, 1 m*l* of incomplete DME was added slowly for the first 1 min and 2 m*l* was added for the next 1 min. For a period of 10 min, a total of 20 m*l* of incomplete DME was added. Cells were collected by centrifugation for 1 min at 650g, suspended in 20 m*l* of HAT (hypoxanthine, aminopterin, and thymine) medium carefully by swirling, and centrifuged for 1 min at 650g. The cells were resuspended in 120 m*l* of HAT medium and 1 m*l* of cell suspension was transferred into each well of five 24-well plates.

Immunoblotting Analysis

For immuno-dot blotting, Small squares (1×1 cm) were drawn on a sheet of nitrocellulose paper (10×10 cm) and marked by numbering. One microliter of antigen solution (1 mg/m*l*) was applied onto each square and air-dried. The blots were incubated for 1h in Blotto (2% non-fat dry milk in TBS), rinsed briefly with TBS, and air-dried. The blots were processed by the procedures described in Western blotting except the substrate. In dot blotting 4-chloro-1-naphtol was used for color rection instead of a chemiluminescent substrate for convenience.

For Western blotting, proteins separated by SDS polyacrylamide gel electrophoresis were electrophoretically transferred to nitrocellulose membranes (Towbin *et al.*, 1979), rinsed briefly in distilled water, and air dried. The blots were blocked with 2% non-fat dry milk in TBS for 1 h. After rinsing with TBS, the blots were incubated in culture supernatants for 1 h and washed 3 times in TBS-Tween 20 at 5-min intervals. The blots were treated with alkaline phosphatase-conjugated goat anti-mouse IgG(Jackson ImmunoResearch) for 1 h and washed 3 times at 5-min intervals with TBS-Twen 20. Following the final rinse for 5 min with an alkaline phosphatase buffer (100 mM Tris-HCl and 5 mM $MgCl_2$, pH 9.5), the blots were overlaid with 1 m*l* of chemiluminescent substrate (LumiPhos 530, Boehringer Manheim Germany), placed in transparent plastic bags, and squeezed with fingers to remove excess amount of the substrate solution. After 10 min, the blot was exposed to x-ray film for 1 h at room temperature.

Other methods

Native polyacrylamide gel electrophoresis was performed according to the procedure of Davis (1964). Discontinuous SDS polyacrylamide gel electrophoresis was carried out as described by Laemmli (1970). Molecular mass of the native enzyme was measured by a FPLC Superose-12 gel filtration column in 20 mM potassium phosphate, pH 7.2, containing 0.05 M NaCl. Spectrophotometric measurements were caried out with a Kontron UVIKON 930 Model double beam spectrophotometer.

Table I. Purification of bovine brain succinic semialdehyde reductase

Steps	Proteins (mg)	Activity (units)	Specific Activity (miliunits/mg)	Yield (%)
1. Crude Extracts	245,000	189	0.77	100
2. Ammonium Sulfate	47,400	59	1.24	31
3. CM-Sepharose	25,405	47	1.85	25
4. Blue-Sepharose	1,310	34	26	18
5. Hydroxyapatite	60	15	250	8
6. Mono-Q	8	9	1,125	5

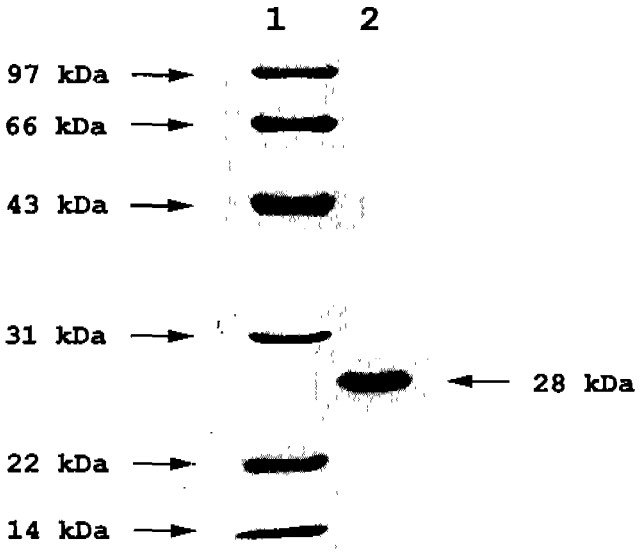

Fig. 1. SDS-PAGE of bovine brain succinic semialdehyde reductase. The gel electrophoresis was performed on 12% separating gel and 4% stacking gel and the gel was stained with Coomassie blue. Lane 1: molecular wight standard. lane 2: purified succinic semialdehyde reductase.

Results and Discussion

The results of a typical purification procedure are shown in Table I. The purification was exhibited 1,500 fold with a recovery yield of 5%. The enzyme purified through the FPLC Mono-Q step exhibited one protein band on a SDS polyacrylamide gel and a native polyacrylamide gel. The enzyme was a monomeric protein with molecular masses of 29,000 and 28,000 as determined by FPLC Superose-12 gel filtration column and SDS polyacrylamide gel electrophoresis, respectively (Fig. 1)

Succinic semialdehyde reductase was reacted with various concentrations of diphenylhydrantion, one of known anticonvulsant drugs (Bronaugh and Erwin, 1973). The effect of diphenylhydantoin on the enzyme

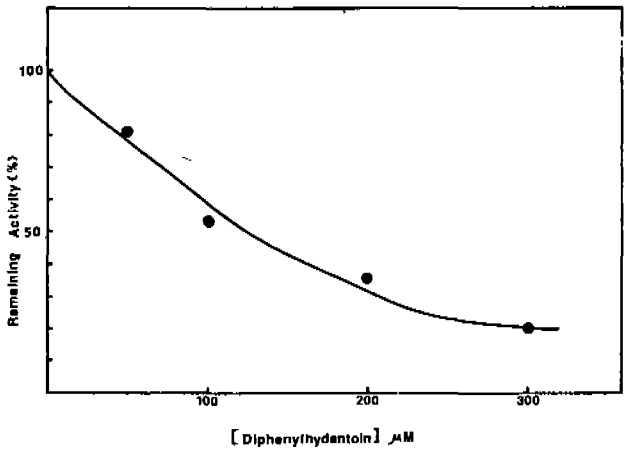

Fig. 2. Inhibition of succinic semialdehyde reductase by an anticonvulsant drug, diphenylhydantoin. The enzyme (10 μM) was preincubated with various concentrations of diphenylhydantoin (50, 100, 200, and 300 μM) in 50 mM potassium phosphate buffer (pH 7.0) at 25℃.

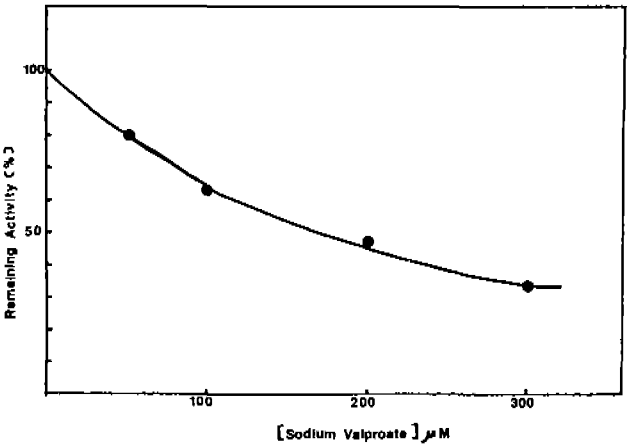

Fig. 3. Inhibition of succinic semialdehyde reductase by anticonvulsant drug, sodium valproate. The enzyme (10 μM) was preincubated with various concentrations of sodium valproate (50, 100, 200, 300 μM) in 50 mM potassium phosphate buffer (pH 7.0) at 25℃.

activity was investigated by preincubating the enzyme (10 μM) with various concentrations of diphenylhydrantoin at 25℃ in 0.1 M phosphate buffer (pH 7.0). As shown in Fig. 2, approximately 80% of the sucinic semialdehyde reductase activity was lost at the highest concentration (300 μM). Another anticonvulsant drug, sodium valproate, also inhibited the enzyme activity when the enzyme (10 μM) was preincubated with different concentrations of sodium valproate (Fig. 3). Sodium valproate (Epilim) is an effective anticonvulsant both in clinical and experimental epilepsies (Simler *et al.*, 1973; Simon and Penry, 1975; Anlezarn *et al.*, 1976) and administration of this compound has been

Table II. The effects of anticonvulsant drugs on succinic semialdehyde reductase

Anticonvulsant drugs	Concentration	% Inhibition
Diphenylhydantoin	3×10^{-4}	80
Sodium valproate	3×10^{-4}	66
Barbiturate*	3×10^{-4}	0
Chlorpromazine*	3×10^{-4}	0

*specific inhibitor of alcohol dehydrogenase.

shown to raise the cerebral GABA level (Godin *et al.*, 1969). Studies of sodium valproates for the elucidation of the possible mechanism of action have suggested that it may function as an inhibitor of the enzymes in GABA shunt, GABA transaminase and succinic semialdehyde dehydrogenase (Sawaya *et al.*, 1975). However, this inhibition turned out to be relatively weak, showing inhibition constants (Ki values) in the millimolar range from rat brain. The Ki value for SSA dehydrogenase by valproate was 4.8 mM (Harvey *et al.*, 1975) and the inhibition of GABA-T by valproate have been varied, with Ki values ranging from 1.4 mM (Simer *et al.*, 1973) to 9.3 mM (Maitre *et al.*, 1974). The Ki for the GABA-T from human brain has been reported to be as high as 40 mM (Maitre *et al.*, 1978). From our studies, since valproate inhibits more efficiently the catalytic activity of SSA reductase than those of GABA-T or SSA dehydrogenase, the inhibition of the reductase by valproate may be an important site of action of this drug *in vivo*.

In contrast to diphenylhydantoin and sodium valproate, barbiturate and chlorpromazine failed to inhibit any enzyme activities, though barbiturate and chlorpromazine were found to be an inhibitor of the enzyme from hamster and monkey brain (Table II). These results suggests that the microenvironments of the catalytic site on the succinic semialdehyde reductase from bovine brain may be different from those of hamster or monkey brain, while the precise structures of the proteins remain to be known. Experiments are already under way to investigate the structural properties of the catalytic domain on the SSA reductase purified from bovine brain. It was reported that chlorpromazine (0.5 mM) had no inhibitory effects on the succinic semialdehyde reductase isolated from human and hamster brains (Cash *et al.*, 1979; Kaufman *et al.*, 1979), and we also obtained similar result.

From the fusion experiments, 15 positive clones were initially selected by dot blotting analysis. Among the clones, some clones lost the ability to produce mAbs and thus were discarded. Nine hybridomas out of the 12 clones were found to secrete the antibodies

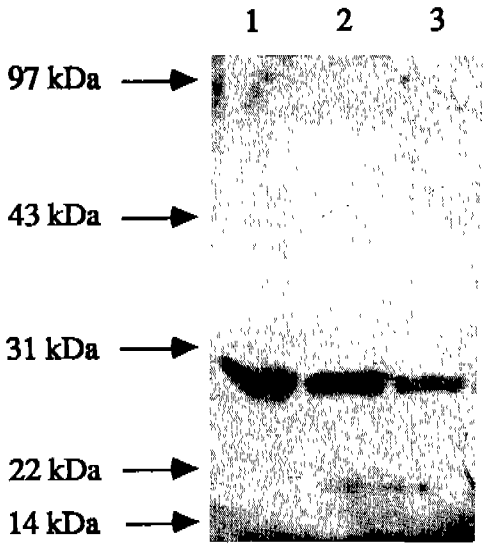

Fig. 4. Immunoreactivities of monoclonal antibodies to succinic semialdehyde reductase. Purified enzyme was separated on a 12% SDS polyacrylamide gel and the blots were treated with culture supernatant and secondary antibody as described in Methods. Lumiphos 530 was used as a chemiluminiscent substrate and the blots were exposed to X-ray film for 1 h at room temperature. The monoclonal antibodies recognized polypeptide band of 28 kDa. Lane 1, mAB SR36; 2, mAb SR136; 3, mAb SR95.

continuously and were further characterized by western blotting. Since the native proteins were used as an immunogen, 6 antibodies showed strong reactivities and the rest did relatively weak color reaction on the western blot, indicating that they reacts with intact antigenic determinants. It is well known that proteins are denatured in the presence of SDS and by heat treatment. The immunoreactivities of some representative mAbs are shown in Fig. 4. The characterization of the mAbs is in progress, and we have undertaken the studies to investigate the expression patterns of the enzyme in brain tissues and to screenn a bovine brain cDNA library using the mAbs as specific probes.

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