SPECIFIC AND NON-SPECIFIC SUCCINIC SEMIALDEHYDE REDUCTASES FROM RAT BRAIN: ISOLATION AND PROPERTIES

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1. Introduction

Succinic semialdehyde (SSA) is an intermediate of the 4-aminobutyrate shunt pathway (GABA-shunt). In mammals, this pathway is particularly important in the brain [1]; this was bypass calculated to account for 10% of the flux through the Krebs cycle [2]. In brain, SSA is primarly oxidized to succinate by a specific dehydrogenase which has been purified and characterized from several species (reviewed [3]). However, brain tissue can also reduce SSA to γ-hydroxybutyrate (GHB) [4] and recently the enzyme(s) responsible have been identified as NADPH-dependent aldehyde reductases [5]. The biosynthesis of GHB in brain tissue is of great interest as this compound which occurs naturally in the brain [6] induces anaesthesia when administered to man and animals in relatively large doses [7]. Although the degree to which this reductive pathway operates in vivo is not as yet known, the recent isolation from human brain of a fairly specific SSA reductase [8] and these similar results using rat brain as the enzyme source, strongly support the hypothesis that GHB biosynthesis may be a significant pathway of pharmacological interest.

2. Materials and methods

Biogenic aromatic aldehydes were obtained by incubation of the parent amines with rat liver monoamine oxidase [9].

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2.1. *Enzyme assays*

For qualitative assays of column eluates, 50 μl enzyme samples were pipetted into tubes of cold 100 mM potassium phosphate buffer (pH 7), containing 5 mM 2-mercaptoethanol and 2×10^{-4} M SSA or *p*-nitrobenzaldehyde. The reaction was started by rapid addition of NADPH to 5×10^{-5} M final conc. in a 1 ml total vol. and subsequent incubation for 30 min at 37°C. Then the decrease in NADPH fluorescence in the samples was measured at excitation 355 nm and emission 470 nm.

Quantitative enzyme assays were performed by direct recording of the initial rate of NADPH oxidation at 37°C in a double beam spectrophotometer at 340 nm, assuming an absorbance of 6.22×10^3 for NADPH. The volume and composition of the incubation medium were identical to that described for the qualitative assay, except that the pH was 6.0 and the reference cuvette contained no aldehyde substrate. The reaction was started by addition of enzyme.

2.2. *Enzyme extraction and purification*

Adult Wistar rats (50) were stunned, decapitated and the brains rapidly removed and suspended in 400 ml final vol. of cold 2 mM potassium phosphate buffer (pH 7) containing 1×10^{-4} M glutathione and 1×10^{-4} M phenylmethylsulphonyl fluoride.

All subsequent operations were done at ~4°C. The suspension was homogenized at maximum speed for 3 min in a food blender. The homogenate was then centrifuged for 1 h at 30 000 × *g* and concentrated KCl solution was added to the resultant clear supernatant solution to 100 mM final conc.

2.3. *Column chromatography*

All rinsing and elution buffers contained 5 mM 2-mercaptoethanol and 5 mM potassium phosphate (pH 7.2) up to the two final chromatographic steps where the pH was 7.8. The supernatant was absorbed onto a 1.6 × 13 cm column of blue Sepharose (Pharmacia). The column was rinsed with phosphate buffer (pH 7.2) containing 100 mM KCl, and then with the same buffer minus the KCl. The outlet of the blue Sepharose column was then attached to a 1.6 × 14 cm column of DEAE-cellulose (Whatman DE 52). The blue Sepharose column was then eluted directly onto the DEAE-cellulose column with 100 ml of 1×10^{-4} M Cibacron blue (Ciba-Geigy) dissolved in the above rinsing buffer. The dye is firmly retained as a narrow band at the top of the DEAE-cellulose column. The DEAE-cellulose column was separated from the blue Sepharose column and rinsed with buffer before elution with 400 ml linear gradient of 0–200 mM KCl in the same buffer. Fractions of 6 ml were collected. It is at this stage that two succinic semialdehyde reductases are separated and thus they will now be referred to as SSR 1 and SSR 2 which is the order in which they are eluted from the DEAE-cellulose column. Both enzymes were then treated separately but almost identically. The enzymes were concentrated to ~10 ml in an Amicon cell equipped with a PM 10 membrane, then diluted to ~100 ml and reconcentrated and rediluted in order to lower the KCl concentration. They were then adsorbed onto 1.6 × 7 cm columns of QAE Sephadex (Pharmacia) columns. After rinsing with starting buffer, enzyme SSR 1 was eluted with a linear gradient of 200 ml 0–100 mM KCl, and enzyme SSR 2 with the same volume of 50–250 mM KCl. Fractions of 4 ml were collected. The final two chromatographic steps were carried out at pH 7.8 and were identical for each enzyme. The active fractions from the QAE Sephadex columns were concentrated to 5 ml and applied to a 2.6 × 100 cm column of Sephadex G-150 (Pharmacia). Elution was carried out with potassium phosphate buffer 5 mM (pH 7.8) containing 5 mM 2-mercaptoethanol and 6 ml fractions were collected. The active fractions were adsorbed directly onto a 0.9 × 16 cm column of 2′,5′-ADP Sepharose (Pharmacia). After rinsing with potassium phosphate buffer 5 mM (pH 7.8) containing 1×10^{-4} M glutathione containing in addition 1×10^{-4} M glutathione, the enzymes were eluted with a linear gradient of 100 ml $0–1 \times 10^{-5}$ M NADP and collected in 3 ml fractions. The pooled active fractions were stored at +4°C.

2.4. *Protein determinations*

The Folin method [10] was used up to the DEAE–Sepharose step and thence the densitometry of the stained protein bands on SDS gels. Bovine serum albumin was used as the standard.

2.5. *Molecular weight determinations*

These were determined using appropriate molecular weight markers by SDS–polyacrylamide gels [11] and non-denaturing gels of different polyacrylamide concentrations [12].

2.6. *pH optima*

These were measured for the two enzymes for both the forward and reverse reactions. For the forward reaction the standard reaction mixture was employed in 100 mM potassium phosphate buffers. For the reverse reaction, 100 mM phosphate buffer was used up to pH 8.0 and thereafter 100 mM borate buffers. The substrate concentrations were GHB = 10 mM and NADP = 5×10^{-4} M.

2.7. K_m *values*

For the forward reaction the K_m values for SSA were measured in the standard reaction medium (pH 6) containing 5×10^{-5} M NADP. The K_m values for NADPH were obtained similarly at a SSA concentration of 2×10^{-4} M. K_m values for GHB in the reverse reaction were measured in 100 mM phosphate buffer (pH 8.1) at 5×10^{-4} M NADP and the values for NADP were similarly determined at 1×10^{-2} M GHB.

2.8. *Substrate specificities and inhibition*

For both enzymes, various aldehydes at 2×10^{-4} M were substituted for SSA in the standard reaction medium at pH 6 and the relative initial reaction velocities compared. Potential inhibitors were similarly tested using 2×10^{-4} M SSA as substrate.

3. Results

Fig.1 shows the separation of 3 peaks of aldehyde reductase activity by DEAE-cellulose chromatography; the first peak (SSR 1) is active with both SSA and *p*-nitrobenzaldehyde. The second peak reduced only *p*-nitrobenzaldehyde whereas the third peak (SSR 2) was specific for SSA.

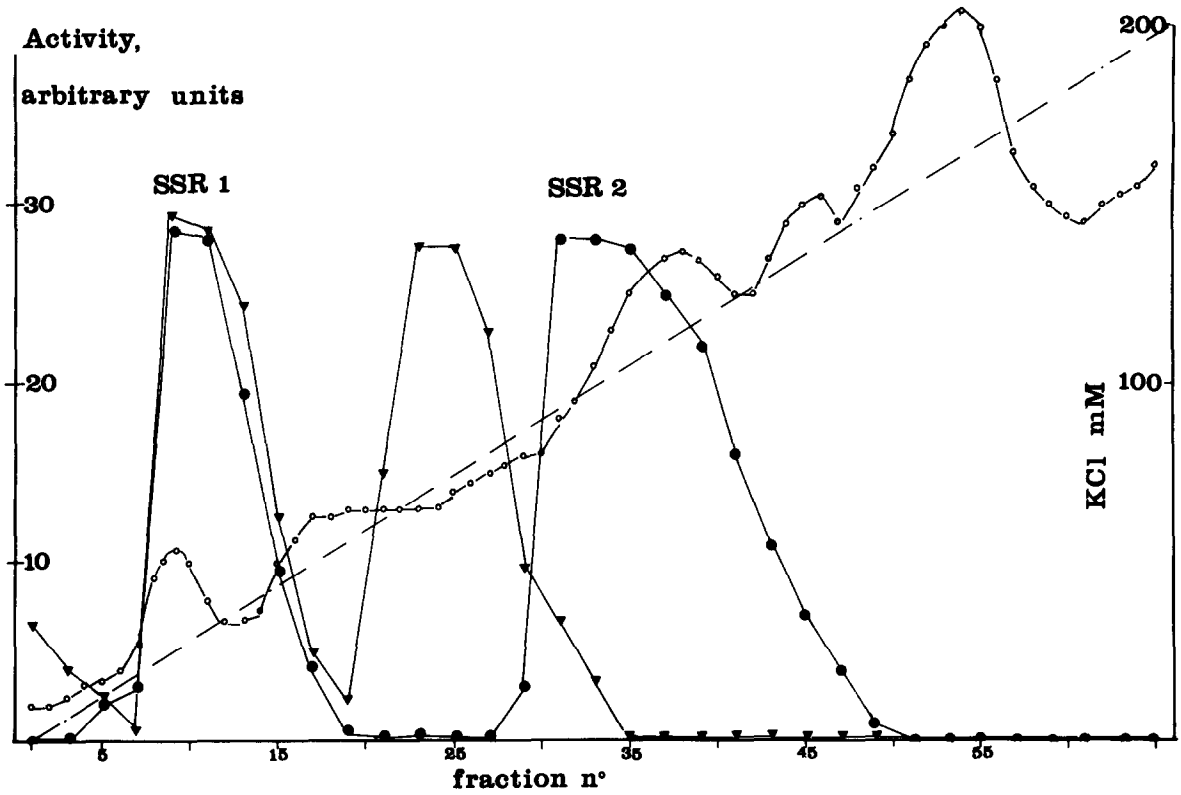

Fig.1. DEAE-cellulose chromatography: (●) Activity with succinic semialdehyde; (▾) Activity with 4-nitrobenzaldehyde; (○) A_{254}

Table 1
Purification of two SSA reductases from 50 rat brains

Fraction	Volume (ml)	Units /ml	Total units	Protein (mg/ml)	Units/ mg protein	Yield (%)	Purification (-fold)
SSR 2							
Supernatant	268	49.2	13 186	9.21	5.34	(100)	–
+ Blue Sepharose DEAE-cellulose	95	26.9	2 556	0.105	256	19.4	48
QAE–Sephadex	59	35.3	2 083	0.0279	1 265	15.8	237
Sephadex G-150	57	19.0	1 083	0.0104	1 827	8.2	342
ADP–Sepharose	10.5	60.3	633	0.0056	10 768	4.8	2 016
SSR 1							
Supernatant	280	56.5	15 820	7.21	7.84	(100)	–
+ Blue Sepharose DEAE-cellulose	50	270	13 500	0.056	4 821	85	615
QAE–Sephadex	50	197.6	9 880	0.017	11 624	62.5	1 483
Sephadex G-150	90	77.6	6 984	0.005	15 520	44	1 980
ADP–Sepharose	18.7	360	6 732	0.020	18 000	42.5	2 297

Units are 10^{-9} mol NADPH oxidized/min at 37°C

Table 1 summarizes the results of a typical purification. Both purified enzymes migrated as single protein bands on SDS and non-denaturing polyacrylamide gels.

The molecular weights determined by SDS gel electrophoresis are, respectively, 50 000 for enzyme SSR 1 and 43 000 for enzyme SSR 2. The molecular weights of the native enzymes determined on polyacrylamide gels are, respectively, 54 000 and 45 000.

The pH optima of enzyme SSR 1 are, respectively, 5.5 for SSA reduction and 8.7 for GHB dehydrogenation (reverse reaction). For enzyme SSR 2, these values are, respectively, 5.0 and 8.1. The K_m values determined under the above conditions are given in table 2. Table 3 shows the relative activities with various aldehyde substrates and table 4 compares the effect of various potential inhibitors on the two enzymes.

Table 2
K_m values expressed in molarities

	SSA	NADPH	GHB	NADP
SSR 1	1.4×10^{-4}	2.6×10^{-6}	1.5×10^{-2}	2.2×10^{-6}
SSR 2	2.8×10^{-5}	2.4×10^{-6}	1.2×10^{-2}	1.4×10^{-6}

Table 3
Substrate specificities

Substrate	Relative activities	
	SSR 2 (Specific enzyme)	SSR 1 (Non-specific enzyme)
Succinic semialdehyde	100	46.5
4-Carboxy benzaldehyde	nd	100
2-Methyl succinic semialdehyde	76.5	85
4-Nitrobenzaldehyde	nd	62
3-Pyridine carboxaldehyde	nd	32
Glyoxal	nd	12.5
D-Lactaldehyde	nd	9.2
3,4-Dihydroxyphenyl-acetaldehyde	nd	8.9
D-L-Glyceraldehyde	nd	3.4
Iso-phthalaldehyde	nd	3.1
5-Hydroxyindolacetaldehyde	nd	nd
Propionaldehyde	nd	nd
Indolacetaldehyde	nd	nd
3-Methoxy-4-hydroxy-phenylglycol-aldehyde	nd	nd
Hydroxyphenylglycolaldehyde	nd	nd
4-Anisaldehyde	nd	nd
n-Valeraldehyde	nd	nd
Phenyl-methyl-ketone	nd	nd
Acetaldehyde	nd	nd
Benzaldehyde	nd	nd
4-Hydroxybenzaldehyde	nd	nd
Glucose	nd	nd
Lactose	nd	nd
Arabinose	nd	nd
Succinic semialdehyde/NADH	20	nd

nd, not detected under our assay conditions

Table 4
Comparative inhibition study

Inhibitor[a]	Percent inhibition	
	SSR 2 Specific enzyme)	SSR 1 (Non-specific enzyme)
Barbiturates		
Barbital	0	80
Pentobarbital	0	76
Phenobarbital	0	72
Amobarbital	0	70
Miscellaneous		
4-Hydroxybenzaldehyde	55	68
Oxalate	0	0
Diphenylhydantoin[b]	0	63
Chlorpromazine	29	43
Pyrazol	0	0
Diazepam	0	25
4-Hydroxybutyrate	20	21
Chloral	0	19
Valproate	10	96

[a] Final conc. 10^{-3} M; [b] Final conc. 2×10^{-4} M

4. Discussion

Enzyme SSR 1 exhibits a broad substrate specificity and is strongly inhibited by barbiturates and certain anticonvulsant drugs. It is thus similar to many NADPH-dependent aldehyde reductases (reviewed [13]). However, enzyme SSR 2 shows a fairly high degree of specificity for SSA, and unlike the first enzyme, it is not appreciably inhibited by the various hypnotics/anticonvulsants tested, including valproate. This enzyme is thus similar to the SSA reductase isolated from human brain [8].

As valproate is an inhibitor of rat brain SSADH [14], its administration might raise the endogenous level of SSA. If this is the case, it could explain the recent finding that administration of valproate to rats brings about some increase in cerebral GHB levels [15] since we have shown that one of the enzymes capable of reducing SSA to GHB is not significantly inhibited by this drug. However, it is unlikely that GHB formation contributes to the anticonvulsant effect of valproate, since administration of this drug to rats has been shown to antagonise the epileptiform electrocorticogram patterns elicited by GHB administration [16].

In view of the multiple effects of GHB on the central nervous system (reviewed [17]) the finding of an enzyme in brain which is apparently specific for its biosynthesis is of great interest.

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