Cloning of a rat brain succinic semialdehyde reductase involved in the synthesis of the neuromodulator γ -hydroxybutyrate

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The γ -hydroxybutyrate biosynthetic enzyme succinic semialdehyde reductase (SSR) was purified to homogeneity from rat brain. Peptides were generated by tryptic cleavage and sequenced. PCR primers were designed from the amino acid sequences of two of the peptides showing a similarity (75–85%) to a mitochondrial aldehyde dehydrogenase. A PCR-amplified DNA fragment was generated from recombinant plasmids prepared by a mass excision procedure from a rat hippocampal cDNA library and used as a probe to screen this cDNA library. One cDNA of 1341 bp had an open reading frame encoding a protein of 447 residues with a deduced molecular mass of 47967 Da. The enzyme was expressed in *Escherichia coli*. Immunoblotting analysis revealed the existence of a protein with the same electrophoretic mobility as the SSR purified from rat brain and with an estimated molecular mass of 45 kDa. Northern blot experiments showed

that this enzyme was not expressed in the kidney or in the liver. In the brain tissue, a single but rather broad band was labelled under high stringency conditions, suggesting the presence of more than one messenger species coding for SSR. Hybridization *in situ* performed on brain tissue slices showed specific labelling of the hippocampus, the upper cortex layer, the thalamus, the substantia nigra, the cerebellum, the pons medulla and the olfactory tract. The recombinant enzyme showed catalytic properties similar to those of the SSR purified from rat brain, particularly in regard to its substrate affinities and K_i for inhibition by phthalaldehydic acid. Valproic acid did not inhibit the cloned SSR. This enzyme had 20–35% identity in highly conserved regions involved in NADPH binding with four other proteins belonging to the aldo-oxo reductase family.

INTRODUCTION

Recent focusing on γ -hydroxybutyrate (GHB) as a substance that can be used in treating heroin or alcohol addiction and withdrawal, but also as a drug taken for psychological benefits, has given a new interest to the endogenous GHB-ergic system [1–6]. This system is potentiated by the peripheral administration of GHB, which penetrates freely into the brain and increases by several orders of magnitude the GHB pool that is naturally present in the central nervous system [7,8]. Endogenous but also exogenous GHB displays important neuromodulatory functions, particularly on the mesocortical and nigrostriatal dopaminergic pathways [9]. This substance is released in brain by neuronal depolarization in a Ca^{2+} -dependent manner [10,11]. GHB binds to a specific class of brain receptors present mainly in the cortical areas of the brain, in the hippocampus and in the dopaminergic regions (striatum, olfactory tracts and dopaminergic nuclei A_9 and A_{10}) [12–14]. Given peripherally at low doses, GHB induces an anxiolytic effect that is accompanied, at higher doses, by sedation and anaesthesia [15–17]. In some categories of animals (rodent, cat, dog or monkey), high doses of GHB can induce petit-mal epilepsy [18–20].

GHB is synthesized mainly in brain by the conversion of γ -aminobutyrate into succinic semialdehyde (SSA), which is then reduced to GHB. A specific succinic semialdehyde reductase (SSR) is responsible for endogenous neuronal GHB synthesis via an NADPH-dependent mechanism [21–24]. SSR shows a high substrate specificity for SSA and is inhibited by phthalaldehydic acid but not by barbituric acid or valproic acid [9]. This enzyme is exclusively located in the cytosol of neurons and in some

neurites and is heterogeneously distributed in the brain [25,26]. The study of the regulation of SSR expression and activity seems to be fundamental to a precise understanding of the functional role of GHB in brain. For these reasons we report the cloning and expression of a cDNA sequence isolated from a rat hippocampus cDNA library that encodes a brain-specific enzyme whose activity characteristics are identical with the SSR activity purified from the same source.

MATERIALS AND METHODS

Materials

Cibacron Blue 3GA agarose, DEAE-Sephadex (3–4 m-equiv./g), diethyl-(2-hydroxypropyl)aminoethyl (QAE)-Sephadex (2.6–3.4 m-equiv./g), NADP agarose, Q Sepharose fast flow, glutathione (reduced form), PMSF, 2-mercaptoethanol, NADPH, isopropyl β -D-thiogalactoside (IPTG), SSA, phthalaldehydic acid and valproic acid were from Sigma (Saint-Quentin Fallavier, France). The modified pig trypsin and PVDF membranes were purchased from Bio-Rad (Ivry sur Seine, France) and the guanidine thiocyanate from Fluka (Saint-Quentin Fallavier, France). Materials for cDNA library construction (Zap Express[®] cDNA synthesis kit, Zap Express[®] cDNA Gigapack II and gold cloning kit) were provided by Stratagene (Montigny-le Bretonneux, France). The *Taq* polymerase and the dNTPs were obtained from Appligene Oncor (parc innovation Illkirch, France), [α -³²P]dCTP from Amersham (ZA Courtaboeuf, Les Ulis, France) and Biotin A membrane from PALL (Portsmouth, Hants., U.K.). T3 and T7 RNA polymerases were

Abbreviations used: GHB, γ -hydroxybutyrate; IPTG, isopropyl β -D-thiogalactoside; QAE, diethyl-(2-hydroxypropyl)aminoethyl; SSA, succinic semialdehyde; SSR, succinic semialdehyde reductase.

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obtained respectively from Stratagene and Promega (Charbonnières, France). Oligonucleotides were synthesized with an Applied Biosystems DNA–RNA Synthesizer Model 1394 with Perkin–Elmer reagents (Norwalk, CT, U.S.A.). Before the nucleotide sequencing on an Applied Biosystems model 373 sequencer, samples were prepared with a Packard Multiprobe 204 DT apparatus and cycled on a Perkin–Elmer 9600 Thermo-cycler. All reagents were from Applied Biosystems. Peptides were isolated by HPLC (Vydac C₁₈ 5m column, 250 mm × 1.6 mm on an Applied Biosystems Microbore apparatus) and sequenced with the Applied Biosystem sequencer model 477A. Sequence analysis and comparison were performed with the Wisconsin sequence analysis package (Genetic Computer). All other reagents were obtained from Sigma Chemical Co.

Purification of SSR from rat brain

SSR was purified by using a previously described method [21,22], with slight modifications. The brains from 50 adult rats were homogenized in phosphate buffer, pH 7.0, and after centrifugation the resulting supernatant was brought to 100 mM KCl and filtered. This preparation was successively subjected to several chromatographic steps, including a blue Sepharose affinity column eluted with 100 µM Blue Cibacron in phosphate buffer, which was directly connected to a DEAE-Sephadex column. After elution with a KCl gradient, the active fractions were pooled and concentrated by means of an Amicon cell concentrator. Then a chromatographic step on QAE-Sephadex was performed, followed by chromatography by gel filtration on an AcA54 column. Finally, SSR was purified to homogeneity with an NADP⁺-agarose affinity column eluted by an NADP⁺ gradient. A single 45 kDa protein band was obtained after SDS/PAGE and staining with Coomassie Blue.

Production of a rabbit polyclonal antibody against SSR

The purified SSR (15 µg in 500 µl of buffer) was mixed 1:1 with Freund's adjuvant and injected subcutaneously into a rabbit once a week for 6 weeks. Blood samples were collected and the antiserum was prepared and kept frozen at –20 °C in 100 µl aliquots until use.

Peptide sequencing

To increase the enzyme purity for peptide sequencing, the SSR purified by the chromatographic steps described above was then subjected to two-dimensional PAGE, which was a combination of an isoelectrofocusing and SDS/PAGE. The SSR was Western blotted on an Immobilon PVDF membrane and located by means of the rabbit anti-SSR polyclonal antibody. The SSR was digested in the gel with a modified pig trypsin [27]; peptides were isolated by HPLC and sequenced.

Rat hippocampal cDNA library

Adult Wistar rats were killed; the hippocampus was rapidly extracted under sterile conditions and frozen rapidly in liquid nitrogen. Total RNA was isolated from the tissue by the guanidinium thiocyanate method [28] and poly(A)⁺ RNA was prepared with an oligo(dT) affinity column [29]. These mRNA species served as a template for the synthesis of single-stranded cDNA species in the presence of a 50-base oligonucleotide primer [designed with a GAGA sequence used to protect a *Xho*I restriction site from nucleases and an 18-base poly(dT) sequence], Moloney murine leukaemia virus reverse transcriptase and nucleotides. The second strand was then synthesized in the presence of RNase H, rat DNA polymerase I and nucleotides.

The double-stranded cDNA was blunt-ended with *Pfu* DNA polymerase. *Eco*RI adapters were ligated to the blunt ends and finally to the Zap Express vector arms. The library was packaged with the DNA *in vitro* packaging set (Stratagene ZAP Express Kits: cDNA Synthesis and Gigapack II).

PCR

PCR primers (5'-GACCGVACMGTTSTTYATYCARCCN-3' and 5'-RAAYTCRTCNACNACRTCYTCYTGNSW-3') were designed on the basis of the amino acid sequences of the two peptides obtained from the trypsin digestion of the purified SSR (see the Results section). PCR was performed with mass excised plasmids of the hippocampal library as matrix (3 × 10⁶ clones, representing ten times the primary library size, were prepared as recommended by Stratagene). The total volume was 100 µl, containing each primer at 1 µM and 1.25 units of *Thermus aquaticus* DNA polymerase in 50 mM KCl/10 mM Tris (pH 8.0)/1 mM MgCl₂/200 µM dATP/200 µM dCTP/200 µM dGTP/200 µM dTTP with the following cycling parameters: 93 °C for 6 min followed by 35 cycles of 94 °C for 45 s, 55 °C for 1 min and 72 °C for 3 min, ending with 72 °C for 10 min (the thermal cycler was a Perkin–Elmer model 480). A single 450 bp DNA fragment was amplified, electroeluted from the agarose gel, extracted with phenol/chloroform and precipitated with ethanol. The 450 bp fragment was then used as a probe to screen the hippocampal cDNA library.

Northern blot analysis

Poly(A)⁺ mRNA was prepared from rat brain hippocampus by using the guanidine/phenol/chloroform method followed by oligo(dT)-cellulose column chromatography [28,29]. RNA samples were incubated with glyoxal at 65 °C for 1 h, resolved in a 1 % (w/v) agarose gel containing 2 M formaldehyde, blotted to a nylon membrane (Schleicher and Schuell) and baked. After prehybridization at 42 °C in the presence of 40 % formamide, the blot was hybridized in the same solution at 42 °C overnight with the PCR-generated 450 bp probe labelled by random priming with [α -³²P]dCTP (6000 Ci/mmol). The blot was washed three times in 0.1 × SSC (1 × SSC is 0.15 M NaCl/0.015 M sodium citrate) containing 0.1 % SDS at 60 °C for 15 min and exposed overnight to an X-ray film (X-omat, Kodak) at –70 °C.

Rat hippocampal cDNA library screening

The cDNA library was plated and screened with the ³²P-labelled 450 bp cDNA obtained by PCR. The library (10⁶ plaque-forming units) was plated on 150 mm NZamine (casein hydrolysate) yeast extract (NZY) agar plates at 50000 plaque-forming units per plate with 600 µl of X11-Blue MRF' and 8 ml NZY top agarose and incubated for 10 h at 37 °C. The plates were chilled for 1 h at 4 °C and phage plaques were transferred to Biodyne A nylon membranes for 5 min. The membranes were then submerged in a mixture of 1.5 M NaCl and 0.5 M NaOH solution for 5 min, neutralized for 5 min in 1.5 M NaCl/0.5 M Tris/HCl (pH 8.0) and washed with a mixture of 0.2 M Tris/HCl, pH 7.5, and 2 × SSC buffer solution. The membranes were baked at 80 °C for at least 2 h before prehybridization in 100 ml of 5 × SSC buffer containing 20 mM NaH₂PO₄, 0.4 % SDS, 5 × PAF (polyvinylpyrrolidone/BSA/Ficoll) and 500 µg/ml denatured salmon sperm DNA for 6 h at 60 °C. The hybridization step was performed at 60 °C overnight in the presence of the labelled probe (10⁶ c.p.m. per ml) with gentle shaking. Putative positive plaques were isolated and rescreened at lower densities until the plaques were purified.

Expression of SSR in *Escherichia coli*

The positive recombinant clones were expressed in XL0LR, a non-suppressing *E. coli* strain. Cells containing recombinant plasmid were grown at 37 °C in Lennox L Broth medium containing kanamycin (50 µg/ml) and treated with 2 mM IPTG when the D_{600} reached 0.6. After 3 h the cells were pelleted by centrifugation and resuspended in one-fifth of the culture volume in 10 mM phosphate buffer, pH 7.0, containing 0.1 mM glutathione and 0.1 mM PMSF. Cells were lysed by freezing and thawing followed by sonication for 10 s in ice, centrifuged at 105000 *g* for 20 min and the supernatant was collected.

Localization of SSR mRNA in the rat brain by hybridization *in situ*

DNA template preparation and transcription *in vitro*

Purified recombinant plasmid DNA (10 µg) was completely linearized with either *EcoRI* or *XhoI* before transcription. *EcoRI* cuts at the end of the insert opposite to the T7 promoter generating the anti-sense transcript, whereas *XhoI* cuts at the end of the insert opposite to the T3 promoter and generates the sense transcript. At the end of the incubation the digestion medium was made up to 100 µl with sterile water. The restricted DNA was extracted with phenol/chloroform and precipitated by the addition of 10 µl of 3 M sodium acetate and 2.5 vol. of ethanol. After centrifugation for 20 min at 20200 *g* in a refrigerated microcentrifuge, the pellet was washed with 1 ml of 70 % (v/v) ethanol, mixed, centrifuged again for 15 min and dried. The transcription reaction was performed in a total volume of 25 µl containing 5 µl of 5× transcription buffer, 1 µg of restricted DNA, 1 µl of 10 mM rATP, 1 µl of 10 mM rUTP, 1 µl of 10 mM rGTP, 1 µl of 1 mM rCTP, 1 µl of 1 M dithiothreitol, 1 µl of RNase block inhibitor, 5 µl of [α -³⁵S]CTP (800 Ci/ mmol), 10 units of T3 or T7 RNA polymerase and diethyl pyrocarbonate-treated water at 37 °C for 30 min. The DNA template was removed by incubating the transcript with 10 units of RNase-free DNase/µg of DNA at 37 °C for 15 min. The RNA probe was extracted with phenol/chloroform and precipitated with ethanol in the presence of 3 M sodium acetate.

Hybridization *in situ*

Rats were anaesthetized with ketamine (100 mg/kg intra-peritoneally) and perfused through the heart with 150 ml of 1 % (w/v) paraformaldehyde at 0 °C. The brains were extracted, immersed for 1 h in the same fixative at 4 °C and then in 15 % (w/v) sucrose for 20 h. After being frozen in liquid isopentane at -40 °C, the brains were stored at -80 °C until used. Un-embedded frozen brains were sectioned (12 µm) with a cryostat and the sections (sagittal and frontal) were thaw-mounted on gelatin-coated slides. Sections were subsequently stored at -80 °C until the hybridization was performed. The pre-hybridization step was performed as follows. The sections were warmed to room temperature for 10 min then placed in 0.25 % (v/v) acetic anhydride in 0.1 M triethanolamine/0.9 % NaCl (pH 8.0) for 10 min. After two rinses in 2× SSC and a 10 min incubation in 50 % (v/v) formamide/1× SSC at 60 °C, the slides were dehydrated by being passed through graded ethanol baths (50 %, 70 %, 90 % and 100 %, v/v) before being air-dried. For hybridization, labelled probes were added to a medium containing 50 % (v/v) formamide, 0.6 M NaCl, 10 mM Tris, pH 7.5, 1 mM EDTA, 1× Denhardt's solution (0.02 % Ficoll/0.02 % polyvinylpyrrolidone/0.02 % BSA), 500 µg/ml yeast tRNA, 250 µg/ml denatured herring sperm DNA, 1 mM dithiothreitol and 10 % (v/v) dextran sulphate. A volume of 35 µl containing approx. 30000 c.p.m. of the probe was added to each slide and

Figure 1 Northern blot of total RNA isolated from rat brain, kidney and liver

The blot was probed with the ³²P-labelled 450 bp PCR fragment; 28 S and 18 S rRNA were used as size markers. Lane 1 corresponds to brain RNA; RNA species from kidney and liver were run in lanes 2 and 3 respectively.

Table 1 SSR activity of the clones isolated (cl1 to cl7)

Only cl4 exhibited an activity that was significantly different from the background (**P* < 0.01). Results are expressed as means ± S.D. (*n* = 3 determinations for each clone).

Clone number	SSR activity (pmol/min per µg of protein)
1	2.80 ± 1.30
2	3.00 ± 1.36
3	5.60 ± 3.00
4	60.50 ± 5.60*
5	1.50 ± 0.80
6	3.60 ± 2.40
7	2.80 ± 1.35

the sections were hybridized under silicone-treated coverslips sealed with Gluck rubber for 16 h at 52 °C in a humid environment. After hybridization was complete, the coverslips were removed and the sections were washed twice with 50 % (v/v) formamide/1× SSC at 55 °C, for 1 h each time, followed by a 30 min incubation in NTE (0.5 M NaCl/5 mM EDTA/10 mM Tris) containing 3 µg/ml RNase A at 37 °C. Finally, the sections were washed twice in 50 % (v/v) formamide/2× SSC at 55 °C for 1 h each time followed by a last wash with 0.1× SSC for 15 min. The sections were dehydrated in graded ethanol baths and air-dried before being exposed to a β-max hyperfilm (Amersham) for 3–7 days at room temperature. Control sections were performed with sense RNA probes.

Western blot and immunodetection

Approx. 20 µg of protein expressed in *E. coli* were separated by SDS/PAGE [10 % (w/v) gel] [30] and transferred to an Immobilon PVDF membrane. After being incubated in 50 mM PBS containing 2.5 % (v/v) skimmed milk at 37 °C for 1 h, the membrane was washed five times with 50 mM PBS. Then incubation with the polyclonal rabbit anti-SSR (diluted 1:500 in 50 mM PBS containing 0.1 % skimmed milk) was performed at

Figure 2 SSR activity of clone c14 expressed in *E. coli*

The enzymic activity was linear up to 10 min of incubation (upper panel) and for 10–150 µg of protein (lower panel).

37 °C for 2 h. The membrane was again washed five times with 50 mM PBS and incubated with peroxidase-conjugated goat anti-(rabbit IgG) (diluted 1:5000 in 50 mM PBS containing 0.1 % skimmed milk) at 37 °C for 1 h. After the membrane had been washed five times with 50 mM PBS, bands were revealed by means of an enhanced chemiluminescence (ECL) kit (Amersham) and exposure to an ECL film (Amersham).

Enzymic assays

The oxidation of NADPH to NADP⁺ was measured by the decrease in absorbance at 340 nm by the method of Hearl and Churchich [31]. Standard assay mixtures contained 50 µM NADPH, 200 µM SSA and 5 mM 2-mercaptoethanol in 100 mM phosphate buffer, pH 6.0, with 50 µg of expressed protein. The change in absorbance at 340 nm was measured after incubation for 5 min. All assays were performed in triplicate. Controls were conducted without SSA. Velocity and inhibition data were plotted with the use of GRAPHPAD Prism software and the double-reciprocal transformation of the equation $v = V_{\max}[S]/(K_m + [S])$. Protein determinations were performed by the Bradford method (Bio-Rad) with BSA as a standard [32].

RESULTS

Peptides sequences obtained after hydrolysis of purified SSR from rat brain

Two peptides, DRTVFIQP and SQEDVVDEF, were chosen from those obtained because they showed 75 % and 85 %

Figure 3 SSR activity of fusion protein expressed in *E. coli*: effect of 1 mM valproic acid and 1 mM phthalaldehydic acid on SSR activity of c14 after 5 min of incubation

Results are expressed as pmol of NADP⁺/min per µg of protein.

Figure 4 Lineweaver-Burk representation of SSR activity

Effect of 0.5 and 1 mM phthalaldehydic acid after 5 min of incubation with various concentrations of SSA and with 50 µM NADPH. The enzyme activity is expressed as pmol of NADP⁺ formed/min per µg of protein. The figure shows the competitive inhibition of phthalaldehydic acid with respect to SSA. Symbols used: (■) no inhibition; (▲) 0.5 mM phthalaldehydic acid; (▼) 1 mM phthalaldehydic acid.

Figure 5 Double-reciprocal representation of SSR activity

Effect of 0.5 and 1 mM phthalaldehydic acid after 5 min of incubation with various concentrations of NADPH (0–50 µM) and with 200 µM of SSA. The enzyme activity is expressed as pmol of NADP⁺ formed/min per µg of protein. The figure shows the non-competitive inhibition of phthalaldehydic acid with respect to NADH. Symbols used: (■) no inhibition; (▲) 0.5 mM phthalaldehydic acid; (▼) 1 mM phthalaldehydic acid.

Figure 6 Western blot and immunodetection of the expressed SSR fusion protein

Proteins (20 μ g) from uninduced and induced samples were subjected to SDS/PAGE [10% (w/v) gel] and blotted on an Immobilon PVDF membrane. Lanes 1 and 2, Coomassie Blue-stained blots of uninduced and IPTG-induced samples respectively; lane 3, immunodetection of SSR with a rabbit polyclonal anti-SSR as first antibody (diluted 1:500), a peroxidase-conjugated goat anti-rabbit IgG as second antibody (diluted 1:5000), revealed by chemiluminescence. The positions of molecular mass standards (in kDa) are indicated at the right.

similarity to a rat liver mitochondrial aldehyde dehydrogenase (EC 1.2.3.1), a nicotinamide-dependent enzyme [33], and were used to design the corresponding PCR primers.

PCR amplification with the hippocampal cDNA library as matrix

A single 450 bp cDNA fragment was amplified by PCR with the set of designed primers and the mass excised plasmids prepared from the hippocampal cDNA library. This 450 bp fragment was not listed in the Genbank/EMBL database. However, it exhibited 30% similarity to members of the aldo-oxo reductase family (results not shown). This cDNA amplified by PCR was used to probe the hippocampal cDNA library.

Northern blot of total RNA from various origins probed with the 450 bp fragment

For the RNA extracted from rat brain (Figure 1, lane 1), the 32 P-labelled 450 bp probe gave a strong and diffuse signal corresponding to a species of approx. 2.0 kb. Interestingly, no band was observed in RNA preparations obtained from kidney or liver (Figure 1, lanes 2 and 3).

Figure 7 Nucleotide sequence of SSR and the predicted amino acid sequence of the encoded protein

The ATG initiation codon, the TAA stop codon and the putative polyadenylation site (AATAAAA) are underlined. The underlined italicized nucleotides indicate the hybridization positions of the degenerate synthetic 24-mer and 27-mer oligonucleotides used for priming the PCR to generate the 450 bp probe. At the amino acid sequence level, the putative protein kinase C phosphorylation sites are in bold type, the tyrosine kinase phosphorylation site is in bold italic characters, and that for casein kinase II is underlined in bold.

The present results describe the isolation of a rat hippocampal cDNA encoding an SSR whose characteristics are identical with those of the enzyme purified from rat brain [22]. The choice of the rat brain hippocampus as the source of mRNA from which to construct the cDNA library was based on previous reports demonstrating it to be a rich source of SSR [26]. Peptides generated by tryptic digestion of SSR purified from rat brain showed 75–80 % similarity to a rat liver mitochondrial aldehyde dehydrogenase (EC 1.2.1.3). These sequences were chosen because of the probable existence of similarities between nicotinamide-dependent dehydrogenases and reductases. Corresponding degenerate oligonucleotides were then synthesized and used as primers for the PCR amplification. A 450 bp cDNA fragment was obtained and a search for similarity in the Genbank/EMBL database was fruitless, indicating that it could correspond to an undescribed cDNA fragment being a part of a putative SSR. Indeed, at the amino acid level, it showed 30 % similarity to members of the aldo-oxo reductase family [37].

Figure 9 Hybridization in situ of the cloned SSR in frontal (A, B) and sagittal (C) slices of rat brain

Some layers of cortex and hippocampus (pyramidal cells layer CA1) are labelled radioactively, as are the thalamus, olfactory tract and substantia nigra (pars compacta). The striatum shows no radioactivity. At the caudal part of the brain, the pons medulla, geniculate bodies and cerebellum are labelled.

Thus the screening of the hippocampal cDNA library under conditions of relatively high stringency could lead to the isolation of a cDNA encoding a homologous protein. This 450 bp cDNA was used to probe a Northern blot of a preparation of rat brain mRNA under high stringency conditions. A major but diffuse band was revealed with an estimated length in good agreement with the relative molecular mass of the SSR. Although we used stringent conditions, a few minor bands probably representing the transcripts of cDNA with which it shared similarities were detected by the 450 bp probe. More interesting is the fact that no signal was detected on the blot of liver and kidney RNA, indicating that this enzyme was expressed specifically in the brain and not in the peripheral tissues examined. Moreover, neurons, but not glial cells, have been found to be SSR immunoreactive at the optic and electronic microscopic levels [9]. On the basis of these observations, we used this 450 bp fragment as a probe to screen the hippocampal cDNA library. Seven clones were brought to homogeneity but only one (cl4) yielded a catalytically active SSR when expressed in *E. coli*. Furthermore this protein was specifically labelled on a Western blot by SSR polyclonal antibodies produced in a rabbit after injections of purified SSR from rat brain. The apparent molecular mass of this protein was approx. 45 kDa, which was close to that of SSR purified from rat brain [22].

The cDNA sequence of the cloned SSR has an open reading frame of 1341 nt, extending from a putative initiation codon (ATG¹⁰⁶) to a stop codon (TAA¹⁴⁴⁷). The deduced protein has a molecular mass of 47967 Da, which is in agreement with the Western blot analysis. The sequences of the other purified clones were different from that of cl4; however, the regions corresponding to the 450 bp probe were 40–50 % similar, which could explain their being cloned along with cl4. It is noticeable that the nucleotide sequence of the SSR has 63 % identity with the rat SSADH in a short region between G⁵⁹² and A⁶³⁷, even though it

has no functional assignment in terms of catalytic activity. At the amino acid level, multiple comparison of the cloned SSR sequence with those of four other proteins belonging to the aldo-oxo reductase family revealed regions with 20–35 % similarities [35–37]. These similarities are significant because they cover conserved areas of the reductases that are thought to be involved in the binding of cofactors and substrates. The 11 amino acid residues that are strictly conserved in all aldo-oxo reductase family members are found in the primary structure of the SSR (Gly⁵², Gly⁷⁶, Asp⁸⁷, Lys¹²¹, Asp¹⁵³, Pro¹⁶⁰, Gly¹⁸⁵, Asn¹⁸⁸, Pro²¹⁶, Gln²²⁰ and Ser³¹¹). An additional six highly conserved positions are also present in the SSR sequence (Gly⁵⁰, Tyr⁹², Gly⁹⁸, Leu¹⁴⁸ and Arg³¹⁷). Five of these residues are involved in binding NADPH (Asp⁸⁷, Asn¹⁸⁸, Gln²²⁰, Ser³¹¹ and Arg³¹⁷) and three form parts of the active site (Asp⁸⁷, Tyr⁹² and Lys¹²¹) [37,38].

It is widely accepted that members of the aldo-oxo reductase family share more than 43 % identity at the amino acid level. They are monomeric NADPH-dependent enzymes with molecular masses in the range 36–40 kDa [37]. Although the SSR has an aldo-oxo reductase activity profile in terms of its catalytic activity, its amino acid sequence showed that SSR was distantly related to the members of this superfamily. This is not peculiar to SSR because the recent cloning of a rat liver enzyme, aflatoxin B₁-aldehyde reductase, which reduces aflatoxin B₁-dialdehyde, also showed less than 25 % identity with the members of the aldo-oxo reductase family [36–41]. Interestingly, the derived amino acid sequence of SSR showed multiple potential phosphorylation sites for protein kinase C, casein kinase II and tyrosine kinase. However, it remains to be verified whether SSR is phosphorylated *in vivo* and whether this phosphorylation regulates its activity. It is noteworthy that the sequences of the two oligonucleotides designed for PCR amplification were found in the reported cDNA sequence with 100 % identity for one and 90 % identity for the other. Consequently a difference in the

derived amino acid sequence of SSR was observed when compared with the original peptides. Obviously, the cloned cDNA encodes for a closely related SSR species other than that which was originally purified from rat brain. Nevertheless the kinetic properties of the cloned enzyme seemed to be the same as those of the purified enzyme [22]. The existence of brain SSR isoenzymes could explain these results and this family of enzymes might have been co-purified during the series of chromatographic steps. This hypothesis is supported by the fact that the shape of the band obtained after the Northern blot performed to detect the SSR mRNA was rather diffuse, indicating that several mRNA species for SSR probably exist in a crude extract. However, the cloned SSR possesses enzyme activity that was competitively inhibited by phthalaldehydic acid with a K_i of 500 μ M (with respect to SSA) but not by valproic acid [9,21,22]. These characteristics are specific to the SSR that synthesizes GHB. Among SSA reductases that are able to transform SSA into GHB *in vitro*, experiments performed on brain slices showed that SSR was the only enzyme involved in GHB synthesis [9,23]. The SSR enzyme isolated from rat brain possesses a high affinity for SSA and a specific pattern of interaction with phthalaldehydic acid. SSA reductases that are not involved in GHB synthesis are inhibited by valproic acid [9]. This substance is devoid of any effect on the cloned SSR; furthermore the affinities of this enzyme for its substrates (SSA and NADPH) are very close to those measured for the purified enzyme (28 and 2.4 μ M respectively) [22]. The determination of SSR activity in discrete regions of rat brain has shown its heterogeneous distribution; immunocytochemical studies have revealed the particular density of SSR protein in the rat cortex and hippocampus [23]. The results of hybridization studies performed with a cRNA probe show a dense labelling of some layers of cortex and hippocampus, but the thalamus, olfactory tract, substantia nigra, cerebellum and pons medulla seem also to be labelled. In all these regions GHB is present at various concentrations [42] but GHB receptors are absent from cerebellum and pons medulla [14]. In these caudal parts of the brain, GHB is thought to be synthesized for metabolic reasons only and does not possess a functional role in neuromodulation. Curiously, the striatum was not labelled by the RNA probe, although it contains SSR, GHB and GHB receptors [14,23]. This region probably contains SSR terminals devoid of mRNA encoding the enzyme. Alternatively, the striatum expresses an SSR isoform that was not labelled under our conditions. If this hypothesis is correct and if several SSR species exist in the rat brain, the present results of hybridization studies show the distribution of only one species of mRNA. However, the use of a complete cRNA for these experiments lead us to suggest that, if a SSR family of mRNA exists, the existence of strong similarities between them makes their detection probable by the present cRNA probe.

The future cloning of a striatal or hippocampal cDNA library at lower stringency could reveal the existence of a specific SSR family and, especially, of an isoform specifically expressed in the striatum. Modification of GHB synthesis in brain could affect the electroencephalogram pattern of sleep and modify the regulation of dopamine synthesis and release. As dopamine is implicated in several neurological and mental diseases, including drug reward and addiction, the knowledge of the regulation of GHB synthesis in brain could be a tool for selecting new drugs with specific therapeutic effects in these domains.

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