Cysteine Sulfinate Aminotransferase and Aspartate Aminotransferase Isoenzymes of Rat Brain. Purification, Characterization, and Further Evidence for Identity†

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ABSTRACT: Two proteins possessing both cysteine sulfinate aminotransferase and aspartate aminotransferase activities were purified from a rat brain supernatant fraction (cytosolic enzyme) and from a rat brain crude mitochondrial fraction (mitochondrial enzyme). The resulting preparations for both isoenzymes were found to be homogeneous by polyacrylamide gel electrophoresis at pH 8, analytical isoelectric focusing on polyacrylamide gels, sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and immunoelectrophoresis. Molecular weights of approximately 84 000 and 86 000 were determined by Ultrogel chromatography for the cytosolic and mitochondrial isoenzymes, respectively. The proteins appeared to be composed of two subunits of the same molecular weight, 41 000 and 43 400, respectively, for the soluble and the mitochondrial forms as determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. p*I* values of 5.7 and 9.1 were determined by focusing on polyacrylamide gels for the cytosolic and mitochondrial isoenzymes, respectively. The values for the limiting substrate dissociation constants are as follows: (for the supernatant isoenzyme) aspartate, 1.52 mM, with 2-oxoglutarate, 0.60 mM; cysteine sulfinic acid, 25.3 mM, with 2-oxoglutarate, 1.03 mM; (for the mitochondrial isoenzyme) aspartate, 0.46 mM, with 2-oxoglutarate, 3.13 mM; cysteine sulfinic acid, 3.36 mM, with 2-oxoglutarate, 1.14 mM. Effect of temperature and activation energy, heat denaturation, substrate specificity, ion effects, and pH optimum were also determined. Some inhibitors have also been studied. Further evidence is provided to support the hypothesis that cysteine sulfinate aminotransferase and aspartate aminotransferase isoenzymes are properties of a single protein, as based on the following findings: (a) upon copurification of the two activities, either from the soluble or from crude mitochondrial fractions, the ratio of their specific activities remained constant; (b) for each isoenzyme similar chromatographic behavior was observed for both activities; (c) similar patterns of both activities were observed regardless of the properties studied. The possible role of these isoenzymes in vivo is discussed.

Cysteine sulfinate aminotransferase (EC 2.6.1) (CSA-T)[1] catalyzes the conversion of cysteine sulfinic acid (the main precursor of taurine biosynthesis) to pyruvic acid and sulfite. However, the existence of such a specific enzyme has not been established. Purified aspartate aminotransferase (EC 2.6.1.1) (AAT) isoenzymes [for a review, see Braunstein (1973)] were able to utilize, besides their "normal" substrates L-glutamic acid and L-aspartic acid, L-cysteine sulfinic acid (CSA) and other amino acids (Singer & Kearney, 1956; Ellis & Davies, 1961; Novogrodsky & Meister, 1964). Although AAT has been extensively investigated, most of the available information on its purification and its miscellaneous properties has been derived from studies on the liver and heart of various species (Morino et al., 1963; Boyd, 1966; Bertland & Kaplan, 1968; Shrawder & Martinez-Carrion, 1973; Schlegel & Christen, 1974; Orlacchio et al., 1979). As far as we know, only a single report (Magee & Phillips, 1971) describes the molecular properties of rat brain aspartate aminotransferase isoenzymes.

In this paper, we describe a method for purification of two mitochondrial and cytosolic CSA-T isoenzymes from rat brain by following CSA-T and AAT activities. Properties of the highly purified isoenzymes are reported (K_m, V_{max}, molecular weight, immunological properties, substrate specificity, thermic and ionic denaturation, optimal pH, p*I*, and free energy). Each isolated isoenzyme demonstrated both CSA-T and AAT activities, suggesting the identity of AAT and CSA-T isoenzymes. The eventual role of CSA-T isoenzymes is discussed.

Experimental Section

Materials

Whenever possible, chemicals of analytical reagent quality were used and purchased essentially from Boehringer, Mannheim (West Germany), except for L-cysteine sulfinic acid which was obtained from Sigma Chemical Co. Ampholine, Ultrodex, and Ultrogel were from LKB, Uppsala (Sweden). CM-Sephadex, DEAE-Sephadex, and octyl-Sepharose were purchased from Pharmacia.

Methods

Enzymatic Assay. Unless otherwise stated, CSA-T and AAT activities were carried out as previously described (Recasens et al., 1978), except that bovine serum albumin (0.1%) was added to the incubation mixture containing 15 mM CSA or 7.5 mM Asp, 7.5 mM 2-oxoglutarate, Tris-HCl buffer, pH 8.6, and 20 μM PLP. The reaction was linear up to 7 min.

Protein Determination. Protein concentrations were determined with the Folin phenol reagent by using the method of Lowry et al. (1951) after precipitation of the protein with trichloroacetic acid to remove interfering substances.

Isoelectrofocusing, Electrophoresis, and Immunoelectrophoresis. Analytical and preparative isoelectric focusing was performed respectively according to Basset et al. (1978) and Radola (1974). Polyacrylamide gel electrophoresis was carried

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[1] Abbreviations used: Asp, aspartic acid; CSA, cysteine sulfinic acid; Glu, glutamic acid; 2-OG, 2-oxoglutaric acid; AET, aminoethylisothiouronium bromide hydrobromide; EDTA, ethylenediaminetetraacetic acid; PLP, pyridoxal phosphate; AAT, aspartate aminotransferase; cAAT, cytosolic aspartate aminotransferase; mAAT, mitochondrial aspartate aminotransferase; CSA-T cysteine sulfinate aminotransferase; cCSA-T, cytosolic cysteine sulfinate aminotransferase; mCSA-T, mitochondrial aspartate aminotransferase.

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out as described by Maurer (1971). Immunoelectrophoresis was performed on 2% agarose gel (Grabar & Williams, 1953).

Ionic Denaturation. The pure isoenzymes were preincubated at various pH values of the medium buffer, 20 mM (HCl, Sørensen–HCl, sodium acetate, phosphate, Tris-HCl, Sørensen–NaOH, and NaOH), at 20 °C for 15 min or for 1 h and then diluted 1/100 in the 100 mM Tris-HCl, pH 8.6, buffer, used for enzymatic determination. (Sørensen buffer is a mixture of glycine (20 mM) and NaCl (20 mM); HCl or NaOH (20 mM) was added to obtain the pH value required.)

Thermic Denaturation. The enzymes were heated for 15 min at various temperatures in 15 mM Tris HCl buffer, pH 8.6, 20 μM PLP, and 0.1% BSA or in the same buffer plus 5 mM CSA or 5 mM Asp or 5 mM 2-oxoglutaric acid. They were then incubated at 37 °C for 7 min so that the final concentrations of the different substrates were identical with those of the first reaction mixture described in the enzyme assay.

Preparation of Crude Enzyme Extracts. One hundred Wistar rats (200–250 g), bred in our laboratory, were used for each purification. After the animals were killed by decapitation, the brains were kept on ice before being homogenized in Potter-Elvejhem homogenizers in a solution containing 0.25 M sucrose, 100 μM EDTA, 100 μM AET, and 20 μM PLP in a final volume of 450 mL.

The homogenate was centrifuged at 900*g* for 10 min. The supernatant was then centrifuged at 20000*g* for 20 min. The pellet was resuspended in 300 mL of the sucrose–EDTA–AET solution and centrifuged under the same conditions. The pooled supernatant was then centrifuged at 100000*g* for 1 h, and the supernatant was kept at −20 °C until fractionation could be continued to purify the cytosolic enzyme (soluble fraction). The 20000*g* pellet was resuspended in 10 mM Tris-HCl buffer, pH 8.6, and 20 μM PLP, and the resultant suspension was then frozen and thawed successively 3 times before being centrifuged at 100000*g*. This later step was repeated twice. Pooled supernatants (mitochondrial fraction) were used for further steps of purification of the mitochondrial isoenzyme.

Purification of the Cytosolic Isoenzyme. Solid $(NH_4)_2SO_4$ was added slowly to the soluble fraction to 50% of saturation, and the resulting mixture was stirred for 3 h. The precipitate which formed was removed by centrifugation at 100000*g* for 20 min, and ammonium sulfate was again slowly added to 85% of saturation. After centrifugation at 100000*g* for 30 min, the precipitate was resuspended in 15 mL of 10 mM Tris-HCl buffer, pH 8.6, and 20 μM PLP and then dialyzed against the same buffer. The fraction was applied to a column (2.8 × 60 cm) of DEAE-Sephadex previously equilibrated by 10 mM Tris-HCl buffer, pH 8.6, and 20 μM PLP. After application of the enzyme, the column was washed with the same buffer. The enzyme was eluted with a linear gradient of increasing concentrations of NaCl (up to 400 mM). Active fractions were pooled and concentrated by filtration with an Amicon filter (UM 10) and dialyzed against 10 mM sodium acetate buffer, pH 5.3. A 2.8 × 50 column of CM-Sephadex was equilibrated with the sodium acetate buffer. After the enzyme was applied, the column was washed with 100 mM sodium acetate buffer, pH 5.3, and then the enzyme was eluted by a linear gradient acetate buffer (up to 250 mM). Sodium chloride salt was added to the pooled and concentrated fraction to a final concentration of 1 M. Then, this fraction was applied to a 0.9 × 20 cm column of octyl-Sepharose and washed with sodium acetate buffer containing 1 M sodium chloride. The active fractions were then pooled and concentrated. The preparation from the preceding step was applied to an Ultrogel column (3.5 × 120 cm), and the column was washed with 100 mM sodium acetate buffer, and 200 mM NaCl. The fractions containing the activity were pooled, concentrated, and dialyzed against 10 mM sodium acetate buffer, pH 5.3. After addition of 10% glycerol, the enzyme was kept at −20 °C and was stable for several weeks under these conditions.

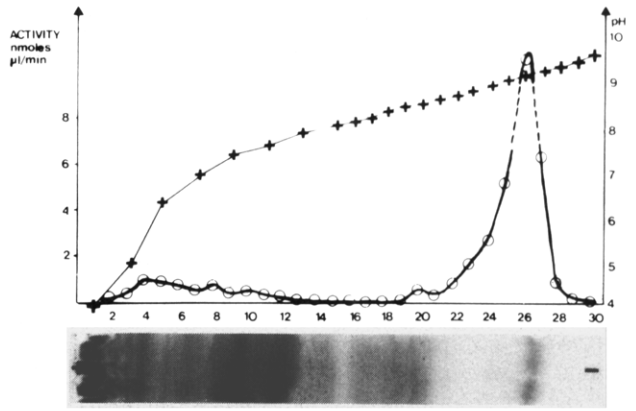

FIGURE 1: Preparative electrofocusing of the ammonium sulfate fractionation (62–85% saturation) of the crude mitochondrial extract. (○) Specific CSA-T activity; (+) = pH for the 30 fractions. The print corresponds to the protein distribution.

The octyl-Sepharose and Ultrogel columns could be replaced by a second CM-Sephadex column (1.5 × 25 cm) at pH 5.3 (sodium acetate buffer), followed by a hydroxylapatite column (1.0 × 25 cm) equilibrated in 10 mM phosphate buffer, pH 8.3. Similar results were obtained.

Purification of the Mitochondrial Isoenzyme. Ammonium sulfate powder was slowly added to the continuously stirred enzyme solution. The precipitate between 62 and 85% of saturation was collected and resuspended in 10 mL of a 10 mM Tris-HCl buffer, pH 8.6, containing 20 μM PLP. The solution was then dialyzed against this buffer for 24 h. Preparative isoelectrofocusing was performed in flatbeds of granulated gel according to Radola (1974). The pH gradient was built with ampholine (L.K.B.) by mixing ampholine 6-8 (1 mL), ampholine 7-9 (2 mL), and ampholine 9-11 (4 mL) for a 100-mL gel. Ultrodex was the supporting medium and glycerol (final concentration 13%) was added to the gel mixture. The dialyzed sample, 6 mL at a protein concentration not exceeding 50 mg/mL, was applied to the middle of the gel after 2 h of prefocusing. Focusing was then performed overnight at 0 °C by using a constant powder of 8 W (Figure 1). The gel fractions from different regions of the gradient were collected and the enzymatic (active) ones were pooled. After removal of the Sephadex gel of the preparative electrofocusing, the solution was concentrated to 6 mL by filtration on an Amicon UM 10 membrane and passed through an Ultrogel 3.5 × 85 cm column equilibrated with 10 mM Tris-HCl buffer, pH 8.6, 20 μM PLP, and 10 mM NaCl. The active fractions were collected, concentrated, and dialyzed against 10 mM Tris-HCl buffer, pH 8.6, and 20 μM PLP. The enzyme was stored at −20 °C after addition of 10% glycerol.

Results

The results of typical purification of the two isoenzymes are summarized in the Table I. The purifications have been conducted 25 times. All operations were quite reproducible. Preparation of the two isoenzymes showed that CSA-T and AAT activities of "crude mitochondrial", on one hand, and "soluble fractions", on the other, were parallel with regard to

Table I: Summary of Purification Procedure for CSA-T Isoenzymes[a]

step	total protein (mg)	total act. (units)	sp act. of CSA-T (or AAT) (units/mg)	ratio of sp act. of AAT/CSA-T	recovery (%)	purifcn (x-fold)
			Cytosolic Enzyme			
100000g supernatant	1730	1055	0.61 (0.77)	1.26	100	
ammonium sulfate precipitation (50–80%)	264	766	2.9 (3.7)	1.28	73	5
DEAE-Sephadex chromatography	47	612	13 (17)	1.30	58	21
CM-Sephadex chromatography	8.3	515	62 (82)	1.32	49	100
octyl-Sepharose chromatography	3.9	437	112 (152)	1.36	41	184
Ultrogel filtration	1.5	243	162 (215)	1.33	23	266
			Mitochondrial Enzyme			
mitochondrial fraction	2280	2234	0.98 (0.31)	0.32	100	
ammonium sulfate precipitation (62–85%)	389	1867	4.8 (1.30)	0.31	84	5
isoelectric focusing	2.7	940	348 (101)	0.29	42	355
Ultrogel filtration	1.1	779	708 (202)	0.29	35	722

[a] A unit is defined as the amount of CSA-T which results in the production of 1 μmol of Glu/min at pH 8.6 and at a concentration of 2-OG of 7.5 mM, of CSA of 15 mM, or of Asp of 7.5 mM.

the further purification steps. This supports the idea that CSA-T and AAT activities are properties of a single protein, regardless of whether it is a mitochondrial or soluble isoenzyme.

The data showed that the cytosolic and mitochondrial isoenzymes were purified respectively 266- and 722-fold, which is in good agreement with the purification previously reported for AAT isoenzymes (Magee & Phillips, 1971). In a previous report (Recasens et al., 1979) a purification of the mitochondrial isoenzyme was described by ammonium sulfate fractionation and DEAE-Sephadex, hydroxylapatite, and octyl-Sepharose chromatography, followed by gel filtration on Sephacryl S-200. The purification described in this paper is more rapid and convenient as three chromatographic steps (DEAE-Sephadex, hydroxylapatite, and octyl-Sepharose) were replaced by isoelectric focusing as the mitochondrial enzyme migrated in the basic part of the pH gradient, where few proteins focus (Malamud & Drysdale, 1978). Specific activity and homogeneity were comparable to those previously obtained.

Criteria of Purity. A single protein band was found after polyacrylamide gradient gel electrophoresis of both purified isoenzymes (20 μg) either in the absence or in the presence of sodium dodecyl sulfate.

Analytical isoelectric focusing gave a single stained band for the mitochondrial isoenzyme, corresponding to the single peak of enzymatic activity and to a p*I* of 9.1. The fresh preparation of cytosolic enzyme showed a single band corresponding to a p*I* of 5.7, although three bands were found with aging of the preparation, the major one corresponding to a p*I* of 5.7 (Figure 2). Antibodies have been obtained against the cytosolic and the mitochondrial isoenzymes. Double immunodiffusion revealed that the fractions at the different steps of purification and purified enzyme all gave single precipitation lines. With the specific antibodies, no cross-reaction was observed between cCSA-T and anti-mCSA-T and vice versa. Immunoelectrophoresis confirmed these results (Figure 3).

Properties. Molecular weights of 84000 ±5000 and 86000 ± 3500, respectively, were determined by gel filtration for cCSA-T and mCSA-T. Both isoenzymes were found to be comprised of two subunits of the same molecular weight, 41000 ± 3000 and 43400 ± 1000, respectively, for the soluble and the mitochondrial form by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (gradient of 10–20%). The ionic denaturation showed that both isoenzymes were irreversibly

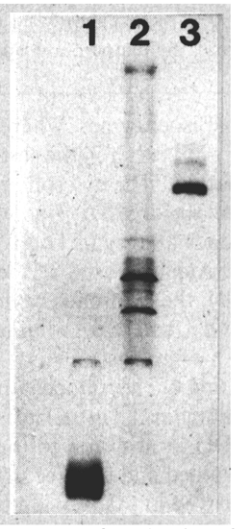

FIGURE 2: Analytical isoelectrofocusing in polyacrylamide gel (1 × 120 × 120 mm) (pH gradient 3–10; temperature 4 °C; constant power 8 W; migration time 2 h; anode at the top). (1) 20 μg of mCSA-T; (2) markers (human hemoglobins and albumin); (3) 11 μg of aged cCSA-T.

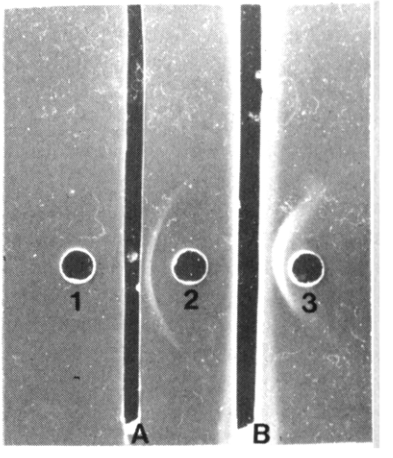

FIGURE 3: Immunoelectrophoresis of 10 μg of enzyme (wells 1 and 3 = purified mitochondrial CSA-T; well 2 = purified cytosolic CSA-T). Lines A and B contained 30 μL of preimmunized sera, anti-cCSA-T, and anti-mCSA-T, respectively.

destroyed at a pH higher than 12 or lower than 2. However, the recovery at pH 3.3 was 90% of the CSA-T or AAT ac-

Table II: Activation Energy[a]

	activation energy (kcal/mol)			
	mitochondrial enzyme		cytosolic enzyme	
pH	CSA	Asp	CSA	Asp
6.3	−9.6 ± 0.5	−11.1 ± 1.0		
			−10.8 ± 1.4	−7.2 ± 0.8
	(−17.3 ± 0.7)	(−20.5 ± 1.8)		
7.2	−9.5 ± 0.8)	−11.9 ± 0.9		
			−10.6 ± 1.1	−8.0 ± 1.0
	(−19.1 ± 1.9)	(−20.8 ± 1.4)		
8.7	−8.7 ± 0.6	−10.2 ± 0.9		
			−9.3 ± 0.8	−6.8 ± 0.4
	(−18.7 ± 1.7)	(−20.4 ± 1.1)		
9.3	−11.3 ± 0.9	−10.4 ± 0.6		
			−10.8 ± 1.2	−6.9 ± 0.6
	(−17.3 ± 1.0)	(−19.8 ± 1.9)		

[a] Activation energies are expressed as kcal/mol and calculated by the formula (Laidler & Peterman, 1979) $E = (4.58 \times 10^{-3})T_2 \times T_1[(\log k_2 - k_1)/(T_2 - T_1)]$, where k_1 and k_2 are rate constants at absolute temperatures T_1 and T_2. Values are means ± SD of five experiments. Numbers and numbers in parentheses for the mitochondrial enzyme correspond to activation energy values calculated from the variation of velocity as a function of temperature between 18.5 and 43 °C and between 0 and 18.5 °C, respectively. For the cytosolic enzyme, the temperature was varied between 0 and 28 °C.

tivities for the soluble isoenzymes, whereas it was ~60% of the CSA-T or AAT activities for the mitochondrial enzyme after 1 h of preincubation. The cytosolic isoenzyme had a pH optimum from 8.5 to 10 for CSA-T as well as for AAT activities. The mitochondrial enzyme had a pH optimum of 8.7 when either CSA or Asp was used as the substrate. At the physiological pH (7.4), the cytosolic enzyme activity was 60% of the maximal activity, whereas the mitochondrial one was 95%.

Thermic denaturation of the mitochondrial enzyme showed that 15 min of preheating in the usual buffer or in the presence of CSA, Asp, or 2-OG in addition to the usual buffer gave the same patterns obtained and that the mitochondrial enzyme was completely destroyed at 70 °C. Up to 50 °C, CSA-T or AAT activities were slightly decreased (10%). The cytosolic enzyme was more thermosensitive. At 50 °C, only 40 and 20% of the maximal activity were recovered for CSA-T and AAT activities, respectively, after preincubation in the usual buffer; addition of 2-OG (5 mM) did not protect the enzyme, whereas addition of CSA (5 mM) protected the enzyme since at 65 °C ~85% of the total CSA-T activity was recovered. Asp did not protect the cytosolic AAT activity from temperature denaturation.

The effect of incubation temperature on activities was investigated at various pH values. This revealed that AAT and CSA-T activities were linear up to 28 °C for the cytosolic enzyme. Two straight lines between 0 and 18.5 °C and between 18.5 and 43 °C were found for the mitochondrial enzyme. At optimal pH, the energy of activation calculated from an Arrhenius plot (the logarithm of the enzyme activity was plotted against the reciprocal of the absolute temperature) was at a minimum, 6.8 ± 0.4 kcal/mol for the cytosolic enzyme, when Asp was used as substrate. Moreover, at each pH studied, the energy of activation was lower for Asp than for CSA (Table II). On the contrary, for the mitochondrial isoenzyme, a minimum energy of activation was obtained, when CSA was used as substrate. Consequently, Arrhenius' law is obeyed by both isoenzymes, suggesting that the velocity is controlled mainly by one rate constant. Arrhenius plots showed a break in the slope occurring at 18.5 °C for the mitochondrial enzyme.

Table III: Substrate Specificity[a]

	rel act. (%)	
substrates	mitochondrial enzyme	cytosolic enzyme
CSA	349 (100)	100
Asp	103 (29.5)	131
cysteic acid	84.1 (26.8)	2.4
α-aminoadipic acid	69.3 (19.9)	20.3
homocysteine	49.5 (14.2)	12.7
2,4-diaminobutyric acid	33 (9.4)	5.8
tryptophan	12.3 (3.5)	2.4

[a] Activities were determined under the conditions described under Experimental Section. Final concentrations in the incubation medium were 15 mM except for tryptophan (4 mM) and tyrosine (1.5 mM). No activity was detectable for the following substrates: methionine, cysteine, hypotaurine, taurine, homocysteic acid, α-aminobutyrate, γ-hydroxybutyrate, GABA, ω-aminocaprylic acid, glycine, alanine, β-alanine, mercaptoethylamine, tyrosine, tryptamine, noradrenaline, dopamine, tyramine, *N*-formylaspartic acid, arginine, lysine, serine, threonine, or homoserine. The relative activities of the mitochondrial and cytosolic isoenzymes are compared with the activity of the soluble enzyme toward CSA taken as 100%. Numbers in parentheses refer to the relative activities of the mitochondrial enzyme (the activity with CSA as substrate taken as 100%).

Substrate specificity is shown in Table III. CSA, Asp, cysteic acid, and to a lesser extent α-aminoadipic acid, homocysteine, tryptophan, and 2,4-diaminobutyric acid were transaminated by the purified mitochondrial isoenzyme. These substrates were also transaminated by the soluble enzyme. In our assay condition, the rate of transamination is more important for CSA than for Asp for the mitochondrial enzyme, whereas this rate is lower for CSA than for Asp with the cytosolic enzyme. Cysteic acid was transaminated at the same rate as Asp by the mitochondrial enzyme, and it was weakly transaminated by the soluble enzyme. All substrates have a common part of their structure: $-CH_2CH(NH_2)COOH$. Although this structural similarity seems to be necessary, it is not sufficient, as cysteine, valine, serine, and other amino acids did not serve as substrates.

The kinetic constants of both isoenzymes were determined by using concentrations of 0.8–7.5 mM CSA and 0.06–0.75 mM 2-OG or 0.5–7.5 mM Asp and 0.2–5 mM 2-OG for the cytosolic enzyme and concentrations of 0.8–10 mM CSA and 0.2–5 mM 2-OG or 0.05–1 mM Asp and 0.5–20 mM 2-OG for the mitochondrial isoenzyme. For the cytosolic enzyme, the dependency of the apparent kinetic constants for CSA, as a function of the concentrations of 2-OG and vice versa, and those for Asp as a function of the concentrations of 2-OG, and vice versa, were fitted to eq 1.

$$\frac{1}{v} = \frac{1}{V}\left[\frac{K_{2\text{-OG}}}{[\text{2-OG}]}\left[1 + \frac{[\text{AA}]}{K_{i(\text{AA})}}\right] + \frac{K_{\text{AA}}}{[\text{AA}]}\left[1 + \frac{[\text{2-OG}]}{K_{i(\text{2-OG})}}\right] + 1\right] \quad (1)$$

Similar data were obtained for the mitochondrial enzyme. v is the measured velocity, depending on the substrate concentrations, V is the maximum velocity under given conditions for both substrates, [2-OG] and [AA] are the concentrations of 2-OG and the amino acid used in the experiments (CSA or Asp), respectively, $K_{2\text{-OG}}$ and K_{AA} are the Michaelis constants for the substrates under saturated conditions, and $K_{i(\text{2-OG})}$ and $K_{i(\text{AA})}$ are the inhibition constants for 2-OG and the amino acids. This relationship was derived from the rate equation which is operative in a ping-pong bi-bi mechanism, where

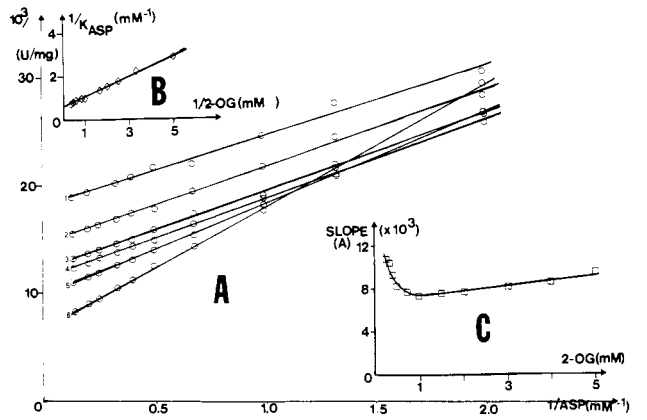

FIGURE 4: (A) Double-reciprocal plot of initial velocities against the concentrations of Asp at a series of fixed concentrations of 2-OG to determine the apparent Michaelis constants of ASP for the cytosolic enzyme. For clarity curves at only some fixed increasing concentrations of 2-OG are given. (1) [2-OG] = 0.2 mM; (2) [2-OG] = 0.3 mM; (3) [2-OG] = 0.4 mM; (4) [2-OG] = 0.5 mM; (5) [2-OG] = 0.6 mM; (6) [2-OG] = 1.25 mM. (B) Double-reciprocal plot of apparent Michaelis constants K_{ASP} [obtained from (A)] against the concentrations of 2-OG to determine the limiting Michaelis constants of the two substrates. (C) Replot of slopes of the lines given in (A) and (B) vs. the concentration of 2-OG to determine the K_i.

competitive substrate inhibition occurs with the two substrates (Cleland, 1963). The values of Michaelis constants for saturated conditions were obtained by replotting the apparent maximum velocities and the apparent kinetic constants as a function of the reciprocal substrate concentrations by the procedure of Velick & Vavra (1962) (Figure 4). Values for the limiting Michaelis constants are summarized in Table IV. As shown in Table IV, we used simplified equations for the calculation of the limiting Michaelis constants. The factors $[2\text{-OG}]/K_{i(2\text{-OG})}$ and/or $[AA]/K_{i(AA)}$ were respectively neglected in front of 1 in eq A, B, C, and D, which were derived from respectively (Garces & Cleland, 1969)

$$\frac{1}{V_{max}^{app}} = \frac{1}{V_{max}}\left[\frac{K_{m(AA)}}{[AA]}\left[1 + \frac{[2\text{-OG}]}{K_{i(2\text{-OG})}}\right] + 1\right] \quad (A')$$

$$\frac{1}{K_{AA}} = \frac{1}{K_{m(AA)}}\frac{K_{2\text{-OG}}/[2\text{-OG}][1 + [AA]/K_{i(AA)}] + 1}{1 + [2\text{-OG}]/K_{i(2\text{-OG})}} \quad (C')$$

Equations B′ and D′ could be deducted from eq A′ and C′, respectively, by replacing AA by 2-OG and vice versa. This error introduced in the method of calculation is negligible compared with experimental variations. For a further verification of the appearance of a ping-pong bi-bi mechanism, a reciprocal plot was constructed by using the velocity vs. the concentration of AA, with a constant ratio [AA]/[2-OG]. A linear relationship was clearly shown at various values of the ratio [AA]/[2-OG]. K_i values were obtained from a replot of the slopes of the double-reciprocal curves (Figure 4) vs. the concentration of CSA, 2-OG, Asp, and 2-OG according to Segel (1975), and the following equations derived from eq 1 were applied to calculate the K_i:

$$\text{slope} = \frac{K_{m(AA)}}{V_{max}K_{i(2\text{-OG})}}[2\text{-OG}] + \frac{K_{m(AA)}}{V_{max}} \quad (2)$$

or

$$\text{slope} = \frac{K_{m(OG)}}{V_{max}K_{i(AA)}}[AA] + \frac{K_{m(OG)}}{V_{max}} \quad (2')$$

K_i values were respectively $K_{i(2\text{-OG})}$ = 0.96 mM and $K_{i(CSA)}$ = 5.1 mM and $K_{i(2\text{-OG})}$ = 17 mM and $K_{i(Asp)}$ = 21 mM for the cytosolic enzyme and $K_{i(2\text{-OG})}$ = 2.5 mM and $K_{i(CSA)}$ = 26 mM

Table IV: Limiting Kinetic Constants of CSA-T Isoenzymes[a]

		cytosolic enzyme						mitochondrial enzyme					
replot	eq	K_{CSA} (mM)	$K_{2\text{-OG}}$ (mM)	K_{Asp} (mM)	$K_{2\text{-OG}}$ (mM)	V_{max} (CSA) (units/mg)	V_{max} (Asp) (units/mg)	K_{CSA} (mM)	$K_{2\text{-OG}}$ (mM)	K_{Asp} (mM)	$K_{2\text{-OG}}$ (mM)	V_{max} (CSA) (units/mg)	V_{max} (Asp) (units/mg)
$1/V_{max}^{app}$ vs. 1/[CSA]	A	25.3 ± 4.1				1700 ± 300		3.10 ± 0.30				695 ± 80	
$1/V_{max}^{app}$ vs. 1/[Asp]	A			1.13 ± 0.09			156 ± 11			0.58 ± 0.06			224 ± 18
$1/V_{max}^{app}$ vs. 1/[2-OG]	C		1.17 ± 0.07		0.54 ± 0.03	1900 ± 180	152 ± 14		1.10 ± 0.12		3.54 ± 0.31	730 ± 95	184 ± 19
$1/K_{CSA}$ vs. 1/[2-OG]	B	22.5 ± 1.8	1.10 ± 0.04					4.15 ± 0.52	1.39 ± 0.10				
$1/K_{Asp}$ vs. 1/[2-OG]	B			1.48 ± 0.17	0.72 ± 0.10					0.48 ± 0.05	2.46 ± 0.32		
$1/K_{2\text{-OG}}$ vs. 1/[CSA]	D	20.2 ± 1.2	0.81 ± 0.07					2.84 ± 0.27	0.93 ± 0.10				
$1/K_{2\text{-OG}}$ vs. 1/[Asp]	D			1.94 ± 0.23	0.53 ± 0.05					0.33 ± 0.04	3.40 ± 0.28		
mean value:		22.6	1.03	1.52	0.60	1800	154	3.36	1.14	0.46	3.13	713	204

[a] The limiting Michaelis constants were calculated from the following equations, which were derived from eq A′, B′, C′, and D′ (see the text). Equation A: $1/V_{max}^{app} = 1/V_{max}[K_{m(AA)}/[AA] + 1]$. Equation B: $1/K_{AA} = 1/K_{m(AA)}[K_{m(2\text{-OG})}/[2\text{-OG}] + 1]$. Equation C: $1/V_{max}^{app} = 1/V_{max}[K_{m(2\text{-OG})}/[2\text{-OG}] + 1]$. Equation D: $1/K_{2\text{-OG}} = 1/K_{m(AA)}[K_{m(2\text{-OG})}/[AA] + 1]$. AA = amino acid substrates, whether CSA or Asp. The values of the limiting constants are the means ± SD of five experiments for the mitochondrial enzyme and three experiments for the soluble isoenzyme. The mean values indicated in the table correspond to the mean of the Michaelis constants obtained by the different methods of calculation.

and $K_{i(2\text{-}OG)}$ = 1.9 mM and $K_{i(Asp)}$ = 263 mM for the mitochondrial enzyme. These data indicated that 2-OG is a good competitive inhibitor for CSA or Asp sites of both isoenzymes, whereas CSA or Asp is a rather weak competitive inhibitor for the 2-OG site. They demonstrated that the double-reciprocal plot of initial velocity against the concentration of one substrate at a series of fixed concentrations of the second substrate did not yield accurately a set of parallel lines (Figure 4).

Comparisons were made of the responses of the two isoenzymes to a selective irreversible inhibitor of AAT enzymes, serine *O*-sulfate (John & Webb, 1969). At a concentration of 1 mM, no inhibition occurred. At 20 mM, a period of inactivation of 46 min was found for CSA-T and AAT activities of the cytosolic enzyme. (The period of inactivation corresponds to the time at which 50% of the initial activity is recovered.) No inhibition was obtained for the mitochondrial isoenzyme. Dipropylacetic acid (20 mM) and vinyl-GABA (20 mM) were without effect, while γ-acetylenic GABA (1 mM) inhibited with a period of 36 and 149 min for the cytosolic and the mitochondrial isoenzyme, respectively.

Discussion

The method developed for the purification of cytosolic and mitochondrial cysteine sulfinate transaminase allows the preparation of these enzymes to a high degree of purity as checked by polyacrylamide gel electrophoresis with and without sodium dodecyl sulfate, isoelectric focusing, and immunoelectrophoresis. The specific activity of rat brain mitochondrial isoenzymes is 708 units/mg (CSA as the substrate) and 202 units/mg (Asp as the substrate) and 162 and 215 units/mg, respectively, for the soluble enzyme. The data we obtained indicated that the purified isoenzymes utilize several substrates, mainly cysteine sulfinic acid and aspartic acid, so it is of interest to know whether one single cytosolic enzyme and one single mitochondrial enzyme support CSA-T as well as AAT activities. We have recently reported that CSA-T and AAT activities are not parallel during rat brain ontogenesis (Gabellec et al., 1978). This could be in agreement with the existence of two distinct enzymes CSA-T and AAT, each enzyme existing as two isoenzymes, a mitochondrial and a soluble one. However, these previous experiments were undertaken with homogenates of brain areas, so that CSA-T and AAT activities were measured as a sum of at least two activities (mitochondrial and soluble). Thus, our results could also be explained by the variations of the proportion between the cytosolic and mitochondrial isoenzymes during ontogenesis as the ratio CSA-T activity/AAT activity is different for each isoenzyme. In the present report, we have shown that AAT and CSA-T activities copurified with the cytosolic isoenzyme prepared from the soluble fraction and the mitochondrial isoenzyme prepared from the crude mitochondrial fraction. The ratios of their specific activities, CSA-T/AAT, remained constant. The purification scheme utilized did not physically separate the two activities on the basis of charge, absorption, size, or hydrophobic properties by chromatoraphy on DEAE-Sephadex, CM-Sephadex, hydroxylapatite, Ultrogel, and octyl-Sepharose. The sensitive analytical techniques used to determine the criteria of purity of the preparations did not reveal a contaminant that could explain the presence of the two activities in the same preparation. Moreover, the variation of AAT and CSA-T activities was parallel for each isoenzyme whatever the properties studied (pH optimum and thermic or ionic denaturation).

Taken together, these results provide strong evidence for the isolation of two highly purified isoenzymes, previously designated as aspartate aminotransferase isoenzymes, which possess both cysteine sulfinate and aspartate aminotransferase activities. These results are in agreement with the work of Yagi et al. (1979) on aspartate aminotransferases from pig heart cytosol and mitochondria and from *Escherichia coli* B. These workers have shown that antisera against the two pig heart AAT isoenzymes and the *E. coli* enzyme inactivated almost completley cysteine sulfinate transamination in the crude extracts of pig heart muscle and *E. coli* B. However, these experiments could also be consistent with the existence of two different enzymes AAT and CSA-T, the antibodies for which cross-react.

Considering the identity of CSA-T isoenzymes and AAT isoenzymes that we demonstrated, we compared our results with those obtained by Magee & Phillips (1971) on rat brain AAT isoenzymes. The specific activities of cytosolic and mitochondrial isoenzymes are in the same range. Values for the molecular weight are also of the same order. However, Magee and Phillips found three forms for the mitochondrial isoenzymes after elution from CM-Sephadex, whereas we found only a single form for the mitochondrial isoenzyme. These differences may be due to the different method of extraction of the mitochondrial crude extract. We used a milder extraction with a hyposmotic shock, whereas Magee and Phillips used an extraction with butanol. The cytoplasmic AAT isolated by Magee and Phillips contained no alternate forms although aging of preparations resulted in the appearance of a second, more rapidly migrating component. Although we found only one form for the cytosolic isoenzyme, subforms appeared during storage. These results are in good agreement with the results of Williams & John (1979) on pig heart cytosolic enzyme, which provides good evidence that the generation of subforms occurred by deamidation, at least in vitro.

As these results provide good evidence that the soluble and mitochondrial purified isoenzymes possess dual activity (CSA-T and AAT activities), the problem of their roles in vivo is raised. Does the mitochondrial isoenzyme act mainly as cysteine sulfinate transaminase and the soluble isoenzyme act mainly as aspartate aminotransferase in vivo? The CSA-T specific activity is 3.5-fold higher than the AAT activity for the mitochondrial enzyme, whereas the CSA-T activity is 0.74 that of the AAT activity for the soluble enzyme. For the mitochondrial enzyme the activation energy is lower with CSA as the substrate than with ASP at physiological pH. The contrary was found for the soluble enzyme. Moreover, transamination of CSA requires a considerably lower 2-OG concentration than that necessary for Asp transamination with regard to the mitochondrial enzyme. This was partially reflected by the ratios of the limiting Michaelis constants, $K_{CSA}/K_{2\text{-}OG}$ = 3 and $K_{Asp}/K_{2\text{-}OG}$ = 0.15. In vivo, it is known that the 2-OG concentration (Greenbaum et al., 1971) is lower than the CSA (Baba et al., 1980) and Asp (Lajtha & Toth, 1973) concentrations. Taking into account these data and our results, it could be concluded that the situation in vivo favors the transamination of CSA over that of Asp by the mitochondrial enzyme. On the contrary, these results indicated that in vivo the cytosolic enzyme is probably more able to catalyze the transamination of Asp than that of CSA.

Moreover, a recent report (Palmieri et al., 1979) indicated that L-CSA enters the mitochondria by a specific exchange against aspartate or glutamate and that L-CSA is transported by the aspartate–glutamate carrier. Thus, although these transaminase isoenzymes show an apparent lack of substrate specificity, our results suggest that, despite their structural

similarities, metabolism of Asp and Glu could be independent from CSA metabolism. This is of interest because these amino acids could play an important neuromodulator and/or neurotransmitter role in the central nervous system.

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Aromatic Retinal Analogues and Their Interaction with Cattle Opsin†

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ABSTRACT: The preparation of seven aromatic analogues of retinal, the isolation and characterization of their geometric isomers, and the interaction of these isomers with cattle opsin are reported. Within certain limitations, it has been demonstrated that stable aromatic rhodopsin analogues can be prepared. In general, the stereoselectivity of isomers of these compounds in their interaction with opsin and the absorption properties of the resultant pigments are similar to those of the parent retinal.

The approach of using structurally modified retinal, whether in the form of geometrical isomerism or chain or ring modifications, for probing for information on structure and binding specificity of opsin has been described in many reports in the literature. The classical work of Wald (Hubbard & Wald, 1952) was followed by more recent work from several different laboratories (Blatz et al., 1969; Kropf et al., 1973; Ebrey et al., 1975; Nakanishi et al., 1976; Arnaboldi et al., 1979; DeGrip et al., 1976; Asato et al., 1978; Kini et al., 1979). It appears that there is a general agreement that within certain limitations, e.g., the longitudinal restriction (Matsumoto & Yoshizawa, 1978), the binding site of opsin is fairly flexible, being able to accommodate retinal isomers of varied geometry and analogues which have undergone much chain and ring modifications. An exception is retinal analogues containing an aromatic end group. As far as we are aware only one report describing results on one system has appeared in the literature (Kropf et al., 1973). A photoisomerized mixture of the simple aromatic aldehyde 9-phenyl-2,4,6,8-nonatetraenal (I)[1] was

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