Characterization of Cerebral Cysteine Sulfinic Acid Decarboxylase. Molecular Parameters and Inhibition Studies

AIMO A HEINÄMÄKI, ANJA K. PERÄMAA and R. SAKARI PIHA

Department of Biochemistry, University of Oulu, SF-90570 Oulu 57, Finland

The molecular properties of cysteine sulfinic acid decarboxylase (EC 4.1.1.29) from calf brain were examined in a crude enzyme preparation. The Stokes radius (42 Å) was determined by gel filtration and the sedimentation coefficient, $s_{20,w}$ (6.1×10^{-13} s) by density gradient centrifugation. Calculation of the molecular weight and frictional ratio gave values of 73 000 and 1.52, respectively.

β -Mercaptopropionic acid and an arginine reagent, phenylglyoxal, were observed to be potent inhibitors of the enzyme. Pyridoxal phosphate, a cofactor of the decarboxylase, was observed to protect the enzyme against phenylglyoxal inhibition. This result indicates that an arginine residue may be located at the active site of cysteine sulfinic acid decarboxylase. The components of taurine metabolism gave little or no inhibition of the decarboxylase. Glutaric acid, malic acid and *C*-allylglycine, all widely known as potent inhibitors of glutamic acid decarboxylase, only slightly inhibited cysteine sulfinic acid decarboxylase.

The molecular properties of cerebral cysteine sulfinic acid decarboxylase (CSAD) are poorly known. In our previous studies taurine-synthesizing CSAD from calf brain was purified to a high degree,^{1,2} but because of the low yield and reduced stability of the pure preparation any extensive examination of molecular properties of cerebral CSAD would have proved laborious. In the present study a crude enzyme preparation was used to achieve some molecular data on CSAD from calf brain. The Stokes radius of the enzyme was determined by gel filtration and sedimentation coefficient by density gradient centrifugation. Both of these methods are applicable to proteins in crude extracts if the proteins do not interact with other components in the mixture.^{3,4} Subsequently estimates were obtained

for the molecular weight and frictional ratio (f/f_0) of the enzyme.

The second part of the work was aimed at examining the effects of some putative inhibitors on the activity of cerebral CSAD. The compounds to be tested were certain components of taurine metabolism,⁵ some inhibitors of glutamic acid decarboxylase (GAD)⁶ and an arginine reagent, phenylglyoxal.⁷ A crude enzyme preparation was also used in these assays.

MATERIALS AND METHODS

Materials. DL-[1-¹⁴C]cysteine sulfinic acid (sp. act. 11 mCi/mmol) was obtained from the Centre d'Energie Atomique (Seclay, France), L-cysteine sulfinic acid, L-cystic acid, aldolase (rabbit muscle), alcohol dehydrogenase (yeast), catalase (bovine liver) and ribonuclease (bovine pancreas) were obtained from Sigma (St. Louis, U.S.A.), pyridoxal-5-phosphate (PLP), taurine, L-cysteine, L-methionine, isethionic acid (2-hydroxyethanesulfonic acid), DL-C-allylglycine, β -mercaptopyruvic acid, malic acid, glutaric acid and phenylglyoxal from Fluka AG (Buchs, Switzerland) bovine serum albumin, egg albumin, α -chymotrypsinogen (bovine pancreas) and 2-aminoethylisothiuronium bromide (AET) from Calbiochem (Los Angeles, U.S.A.), cytochrome *c* (horse heart) from Boehringer Mannheim GmbH (West Germany), Lumagel from Lumac Systems AG (Basel, Switzerland), Sephadex G-200 from Pharmacia (Uppsala, Sweden) and fresh brains of male and female calves from a local slaughter house (Oulu, Finland).

Decarboxylase assay. CSAD activity was measured by collecting the ¹⁴CO₂ from [1-¹⁴C]cysteine

sulfinic acid under anaerobic conditions.² The reaction mixture contained 200 mM sodium phosphate (final concentration) at pH 7.2, 10 mM L-cysteine sulfinic acid, 4.6 kBq of DL-[1-¹⁴C]cysteine sulfinic acid, 0.1 mM PLP, 1.0 mM AET, 0.1 % (w/v) Triton x-100 and 0.5 ml of enzyme solution containing 0.1–2 mg protein from the various enzyme preparations. Total volume of the reaction mixture was 1 ml. The exact performance of the assay proceeded as follows: Purified nitrogen gas was passed through the incubation tube for 1 min, after which the incubation tube containing the reaction mixture was capped with a rubber stopper and a disposable centre well (Kontes Glass Co., Vineland, NJ) containing 150 µl of 40 % KOH to trap the ¹⁴CO₂ produced by the decarboxylation. After incubation at 37 °C for 60 min the reaction was terminated by injecting 0.5 ml 5 N H₂SO₄ through the stopper, whereupon the tubes were allowed to incubate for an additional 30 min. The alkali in the centre well was neutralized with an equivalent amount of HCl. The centre wells were washed on the outside with distilled water to remove any radioactive contamination and then sunk into counting vials (Mill-6, Lumac) containing 5 ml of Lumagel. The vials were shaken well and any chemiluminescence was reduced by allowing them to stand at room temperature for 1 h before counting in a scintillator (1210 Ultrabeta, Wallac LKB). A blank tube was included, containing buffer instead of enzyme.

Decarboxylase preparation. Crude brain decarboxylase was prepared by a three-step method consisting of homogenization of the whole calf brain, acid precipitation and ammonium sulphate fractionation, as described previously.²

Gel filtration. The chromatography column Pharmacia was packed with Sephadex G-200 according to the manufacturer's instructions, and the gel equilibrated with 0.2 M sodium phosphate buffer, pH 7.2, which contained 0.1 mM PLP and 1.0 mM AET. The column was calibrated a series of well-characterized globular protein standards with the following molecular weights (MW) and Stokes radii (a):^{3,18} ribonuclease (MW=13 700, a=16.4 Å); chymotrypsinogen (MW=25 000, a=20.9 Å); egg albumin (MW=43 000, a=30.5 Å); serum albumin (MW=67 000, a=35.5 Å); aldolase (MW=158 000, a=48.1 Å) and catalase (MW=210 000, a=52.5 Å).

Inhibition. The chemicals to be tested were present in the reaction mixture at 10 mM concentrations and the substrate at a concentration of 1.0 mM (*K_m*=1.2 mM).

Density gradient centrifugation. Linear sucrose gradients were produced using an Ultragrad Gradient Mixer (LKB 11300). One mixing chamber containing 32 % (weight per volume) of sucrose in 0.05 M sodium phosphate buffer, pH 7.2, with 0.1 mM PLP and 1.0 mM AET and the other 16 % sucrose (weight per volume) in the same buffer. Two cellulose nitrate centrifuge tubes were filled simultaneously with an identical sucrose gradient using a 2-channel MultiPerpex Pump (LKB 2115).⁸ The linearity of the sucrose gradients was determined by adding Amido Black to the sucrose solution of lower concentration. Following centrifugation and collection of the fractions the absorbance at 400 nm was found to increase in a linear fashion with the fraction number. The gradients were stored in a 4 °C cold room for 5 to 20 h before use.

The protein samples were dissolved individually in 0.05 M sodium phosphate buffer, pH 7.2, with 0.1 mM PLP and 1.0 mM AET, after which 0.2 ml of protein solution containing 5 mg protein was layered on the surface of the 16 % sucrose gradient. The protein standards and the extracts were centrifuged in separate tubes.

Centrifugation was performed for 17 h at 39 000 *g* at 4 °C in the swinging bucket SW-39 rotor of a Spinco Model L ultra centrifuge (Beckmans Instruments).

The sucrose gradient was then unloaded by means of a self-made system consisting of a needle and adjustable stopcock. A hole was punched in the bottom of the centrifuge tube and 0.25 ml fractions were collected manually. The protein content and decarboxylase activity of the fractions were determined.

Calculation of the molecular parameters. The molecular weight (*M*) frictional ratio (*f/f₀*) of the decarboxylase were determined from the classical eqns. (I) and (II).

$$M = 6\pi\eta Ns / (1 - v\rho) \quad (\text{I})$$

$$f/f_0 = a / \left(\frac{3vM}{4\pi N} \right)^{1/3} \quad (\text{II})$$

where η = viscosity of the medium, N = Avogadro's number, a = Stokes radius, s = sedimentation coefficient, v = partial specific volume, ρ = density of the medium, M = molecular weight and f/f_0 = frictional ratio. A partial specific volume of 0.725 cm³/g was used in the calculation, as this value is representative

Fig. 1. A. Distribution of decarboxylase activity (●) and protein (○) on a Sephadex G-200 column (26 × 57 cm). Each sample applied to the column contained 100 mg of protein from the crude enzyme preparation. Fractions of 5 ml were collected; B. The gel filtration data were plotted according to the correlation of Laurent and Killander.¹¹ The standard proteins (●) used are: 1. cytochrome *c*, 2. ribonuclease, 3. chymotrypsinogen, 4. egg albumin, 5. serum albumin, 6. aldolase and 7. catalase. CSAD (○). For details, see Materials and Methods.

of most proteins in sucrose density gradient centrifugation studies.⁴ The values of De Duve *et al.*⁹ were used for the density and viscosity of the sucrose solutions as functions of concentration and temperature.

Protein estimation. Protein content was determined by the method of Lowry *et al.*¹⁰ Bovine serum albumin was used as the standard.

RESULTS

Stokes radius. The apparent Stokes radius was measured by gel filtration using a calibrated Sephadex G-200 column.³ The apparent Stokes

radius of CSAD was estimated to be 42 Å when the gel filtration data were plotted according to Laurent and Killander¹¹ (Fig. 1).

Sedimentation coefficient. The sedimentation coefficient $s_{20,w}$ determined by sucrose density gradient centrifugation according to Martin and Ames,⁴ gave a value of 6.1×10^{-13} s (Fig. 2).

Molecular weight and frictional ratio (f/f_0). The Stokes radius and the sedimentation coefficient obtained by gel filtration and density gradient centrifugation, together with the assumed partial specific volume of 0.725, were used to calculate the molecular weight and frictional ratio (f/f_0) of cerebral CSAD by means of eqns. (I) and (II), respectively. The calculations gave a molecular

Fig. 2. Determination of the sedimentation coefficient of CSAD by density gradient centrifugation: A. Decarboxylase activity in the fractions of the sucrose gradient; B. Relative mobility of alcohol dehydrogenase (ADH),⁴ bovine serum albumin (BSA),³ lysozyme⁴ and CSAD in the sucrose gradients during centrifugation. For details see Materials and Methods.

Table 1. Inhibition of cysteine sulfinic acid decarboxylase.

Compound (10 mM)	Inhibition (%) (mean of 4 experiments)
Components of taurine metabolism	
L-Methionine	0
L-Cysteine	7
L-Cysteic acid	32
Isethionic acid	0
Taurine	0
GAD inhibitors	
Glutaric acid	14
Malic acid	20
C-Allylglycine	15
β -Mercaptopropionic acid	95
Argine reagent	
Phenylglyoxal	93

weight of 73 000 and a frictional ratio of 1.52.

Inhibition of CSAD. Methionine, taurine and isethionic acid produced no inhibition of cerebral CSAD under the conditions mentioned in Materials and Methods. Cysteine, cysteic acid, glutaric acid, malic acid and C-allylglycine caused slight inhibition, whereas β -mercaptopropionic acid and phenylglyoxal were more potent inhibitors. The results of the inhibition studies are summarized in Table 1.

DISCUSSION

A combination of the gel filtration and centrifugation techniques was used here to estimate the molecular weight of cerebral CSAD, from the Stokes radius and sedimentation coefficient, together with a third parameter the partial specific volume, which varies over quite a narrow range for most proteins.⁴ The sedimentation coefficient, Stokes radius and molecular weight of the calf brain enzyme are approximately equal to those for the rat liver enzyme.¹² The frictional ratio was calculated once the values of the above parameters had been established. This shows CSAD from calf brain to be an asymmetric and/or hydrated molecule.

The most potent inhibitor was β -mercaptopropionic acid (95 % inhibition at a concentration of 10 mM), which is widely used as an inhibitor of GAD. It is the mercapto group in this inhibitor which is thought to be important in its binding to the pyridoxal-phosphate enzyme complex,⁶ and it

is plausible that the inhibition mechanism may be the same for CSAD and GAD, since both are pyridoxal-phosphate enzymes.^{6,13} Glutaric acid, malic acid and C-allylglycine also inhibited CSAD activity to a limited extent, whereas they have all been observed to be potent inhibitors of GAD.⁶ The components of taurine metabolism, cysteine and cysteic acid, gave only a slight inhibition of CSAD. The most interesting result is the inhibition of the decarboxylase activity by phenylglyoxal, which is frequently used to detect essential arginine residues at the active sites of enzymes.¹⁴⁻¹⁷ This compound reacts fairly specifically with arginine to form a stable complex.⁷ Pyridoxal phosphate was observed to protect the enzyme against phenylglyoxal inhibition. The results indicate that the arginine residue may be located at the active site of cerebral CSAD.

REFERENCES

1. Heinämäki, A. A. and Piha, R. S. *Acta Chem. Scand. B* 34 (1980) 363.
2. Heinämäki, A. A., Surcel, H.-M. and Piha, R. S. *Acta Univ. Oul. A* 97. *Biochem.* 29 (1980) 61.
3. Siegel, L. M. and Monty, K. J. *Biochim. Biophys. Acta* 112 (1966) 346.
4. Martin, R. G. and Ames, B. N. *J. Biol. Chem.* 236 (1961) 1372.
5. Gaitonde, M. K. In Lajtha, A., Ed., *Handbook of Neurochemistry*, Plenum, New York 1970, Vol. 3, Chapter 8.
6. Taberner, P. V., Pearce, M. J. and Watkins, J. C. *Biochem. Pharmacol.* 26 (1977) 345.
7. Takahashi, K. *J. Biol. Chem.* 243 (1968) 6171.
8. Rosenbaum, T. *LKB Application Note*, LKB-Producter AB, Stockholm 1972, MLB/an-26.
9. De Duve, C., Berthet, J. and Beaufay, H. *Prog. Biophys. Biophys. Chem.* 9 (1959) 357.
10. Lowry, O. H., Rosenbrough, N. J., Farr, A. L. and Randall, R. J. *J. Biol. Chem.* 193 (1951) 265.
11. Laurent, T. C. and Killander, J. *J. Chromatogr.* 14 (1964) 317.
12. Guion-Rain, M.-C., Portemer, C. and Chatagner, F. *Biochim. Biophys. Acta* 276 (1972) 272.
13. Jacobsen, J. G., Thomas, L. L. and Smith, L. H. *Biochim. Biophys. Acta* 85 (1964) 103.
14. Kazarinoff, M. N. and Snell, E. E. *J. Biol. Chem.* 251 (1976) 6179.
15. Cheung, S.-T. and Fonda, M. L. *Arch. Biochem. Biophys.* 198 (1979) 541.
16. Tunncliff, G. and Ngo, T. T. *Experientia* 34 (1978) 989.
17. Tunncliff, G. *Biochem. Biophys. Res. Commun.* 97 (1980) 160.
18. *Gel filtration: theory and practice*. Technical leaflet from Pharmacia Fine Chemicals, Uppsala, Sweden.

Received December 28, 1981.