Biochimica et Biophysica Acta, 321 (1973) 361–368

BBA 66997

L-SERINE DEHYDRATASE FROM RAT LIVER

PURIFICATION AND SOME PROPERTIES

DIETRICH SIMON, JIRO HOSHINO AND HANS KRÖGER
Robert Koch-Institut, Biochemistry Department, Berlin-West (Germany)

(Received March 12th, 1973)

SUMMARY

1. L-Serine dehydratase [L-serine hydrolyase (deaminating), EC 4.2.1.13] from rat liver was purified to electrophoretic homogeneity by a new method.

2. After the last purification step two isoenzymes can be distinguished by DEAE-cellulose chromatography as well as by analytical disc electrophoresis. The same isoenzymes can be demonstrated after electrophoresis of a crude extract from rat liver. The smallest subunit of both isoenzymes has a mol. wt of about 35 000. This is in agreement with previous reports (Inoue, H., Kasper, C. B. and Pitot, H. C. (1971) *J. Biol. Chem.* 246, 2626–2632).

3. From gel filtration experiments in the presence of the substrate L-serine it is concluded that the active form of L-serine dehydratase consists of two subunits. At low enzyme concentrations, the enzyme dissociates into its subunits; K^+ and NH_4^+ counteract this process.

INTRODUCTION

The molecular and enzymatic properties of L-serine (L-threonine) dehydratase [L-serine hydrolyase (deaminating), EC 4.2.1.13] from rat liver have been investigated in different laboratories with partially or highly purified enzyme preparations[1–4]. It has been generally accepted that one single enzyme is responsible for the dehydrative deamination of L-serine and L-threonine, and that the enzyme is free of cystathionine synthetase activity[3]. On the other hand, there is no full agreement about the molecular weight of the enzyme and the existence of isoenzymes. While Nagabushanam and Greenberg[1] reported a mol. wt of 20 000, in our laboratory a value of 42 000 was measured[4]. Nakagawa *et al.*[5], who succeeded in purifying the enzyme to homogeneity found a mol. wt of about 64 000. In agreement with the latter Inoue *et al.*[3], using the same purification method, estimated a mol. wt of 34 000 for the smallest subunit.

Abbreviation: MTT, 3-(4,5-dimethylthiazolyl-2-)-2,5-diphenyl-tetrazoline bromide.

Dedicated to Professor J. Deuticke on the occasion of his 75th birthday.

Concerning the question of isoenzymes, we previously reported another L-serine dehydrating activity in the rat liver, separable from serine dehydratase which we could identify as homoserine dehydratase[6]. Recently Inoue *et al.*[3] characterized an isoenzyme with the same molecular weight as the original L-serine dehydratase.

In the present paper we re-examine the questions of molecular weight, subunit structure and interactions, and the existence of isoenzymes with an enzyme preparation purified by a different method.

MATERIALS AND METHODS

Chemicals

L-Serine, L-threonine, ammoniumperoxodisulfate, riboflavin were obtained from Merck AG. Lactate dehydrogenase (rabbit muscle), cytochrome *c*, fumarase, NADH were from Boehringer Mannheim GmbH; bovine serum albumin, trypsin inhibitor, pyridoxal 5'-phosphate, dithiothreitol, DEAE-cellulose (SH type, 0.84 mequiv/g), acrylamide, *N*,*N*'-methylenebisacrylamide, Coomassie brilliant blue, phenazine methosulfate, 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazoline bromide (MTT), sodium dodecyl sulfate, 2-mercaptoethanol from Serva Feinbiochemica. Pharmacia supplied Sephadex G-100, G-25, and Blue Dextran 2000.

Enzyme assay

L-Serine dehydratase activity was assayed as described previously[4]. 1 enzyme unit is defined as the amount of enzyme, which forms 1 μmole of pyruvate per h at 37 °C.

Electrophoretic procedures

Disc electrophoresis of the pure enzyme was performed in 7% polyacrylamide gels at 2 mA and 4 °C for 2 h as described by Davis[7].

For electrophoresis of the 105 000 $\times$ *g* supernatant of the liver crude extract half the concentration of buffer was used in the separation gel, and the cathodal buffer was made $5 \cdot 10^{-5}$ M in pyridoxal 5'-phosphate.

Gels were stained for L-serine dehydratase activity by incubating them in the dark at room temperature for 15 min, each in 4 ml of a mixture containing 400 μmoles of sodium pyrophosphate·HCl, pH 8.4, 800 μmoles of L-serine or L-threonine, 80 nmoles of pyridoxal 5'-phosphate, 400 μmoles of KCl, 20 mg of MTT, and a few crystals of phenazine methosulfate[8].

For the determination of the molecular weight on sodium dodecyl sulfate gels with or without urea, the procedure of Weber and Osborn[10] was followed.

Sephadex gel filtration

Sephadex gel filtration for the determination of the molecular weight was carried out as described by Andrews[11].

Sucrose density gradients

With sucrose density gradient centrifugation the method of Martin and Ames[12] was followed.

Determination of protein

Protein was determined by the method of Lowry *et al.*[13].

RESULTS

Enzyme purification

Preparation of the crude extract. 60 female Wistar rats (150–170 g) were fed a low protein diet for 7 days and thereafter a high protein diet for the same time. The following procedures, except the heat treatment, were performed at 0–4 °C. After killing the animals, 30-g portions of liver were homogenized with 4 vol. of extraction buffer (0.05 M potassium phosphate, pH 7.2, containing 0.14 M KCl, $1 \cdot 10^{-3}$ M EDTA, $1 \cdot 10^{-4}$ M pyridoxal 5′-phosphate) by an Ultra-Turrax homogenizer at 20 000 rev./min for 75 s. This was followed by centrifugation at 23 000 × *g* for 2 h.

First $(NH_4)_2SO_4$ fractionation. To the supernatant solid $(NH_4)_2SO_4$ was added to 28% saturation (pH 7.5). After centrifugation at 23 000 × *g* for 30 min the resulting supernatant was brought to 45% saturation, the precipitate collected by centrifugation and kept at −18 °C overnight.

Heat treatment. The precipitate was dissolved in 195 ml of 0.05 M potassium phosphate buffer, pH 7.8, containing $1 \cdot 10^{-3}$ M pyridoxal 5′-phosphate and $1 \cdot 10^{-3}$ M EDTA. The solution was heated at 55 °C for 2.5 min (in 5-ml portions in test tubes) and then cooled in an ice–water bath. After this treatment the enzyme was diluted 3-fold with the same buffer and centrifuged.

Second $(NH_4)_2SO_4$ fractionation. The supernatant was diluted to a protein concentration of 8 mg/ml and brought to 43% saturation with solid $(NH_4)_2SO_4$ within 2 h (pH 7.5). After 0.5 h the precipitate was collected by centrifugation and stored at −18 °C.

Gel filtration. Half the precipitate was dissolved in 20 ml of 0.05 M potassium phosphate buffer, pH 7.8, containing $1 \cdot 10^{-4}$ M pyridoxal 5′-phosphate and 10^{-4} M EDTA (Buffer A) and dialysed against this buffer for 2 h. After centrifugation and dilution to a protein concentration of 35 mg/ml, the solution was applied to a Sephadex G-100 column (10 cm × 70 cm) equilibrated with Buffer A. The enzyme was eluted at a flow rate of 200 ml/h, 10-ml fractions were collected. The active fractions were combined and concentrated to 5 ml by ultrafiltration. After treating the second half of the $(NH_4)_2SO_4$ precipitate in the same way, both concentrates were combined, and the buffer was changed to 0.05 M NH_4Cl–NH_3 buffer, pH 8.1, containing $5 \cdot 10^{-5}$ M pyridoxal 5′-phosphate, 10^{-3} M EDTA and 10^{-4} M dithiothreitol (Buffer B) on a Sephadex G-25 column (2 cm × 15 cm).

DEAE-cellulose chromatography. The enzyme solution (150 mg) was adsorbed to a DEAE-cellulose column (2.5 cm × 20 cm) equilibrated with Buffer B. Washing of the column with one column volume of the same buffer was followed by elution with a linear buffer gradient, which was formed with 420 ml of Buffer B in the mixing chamber and with 420 ml of 0.25 M NH_4Cl buffer, pH 8.1, with the same additions as Buffer B in the reservoir. The flow rate was 60 ml/h. 10-ml fractions were collected.

Fig. 1 shows the elution pattern of L-serine dehydratase from the DEAE-cellulose column. Part A was concentrated to 0.5 ml by ultrafiltration, and after addition of 2 ml of Buffer A was stored at 4 °C in the dark. If pyridoxal 5′-phosphate

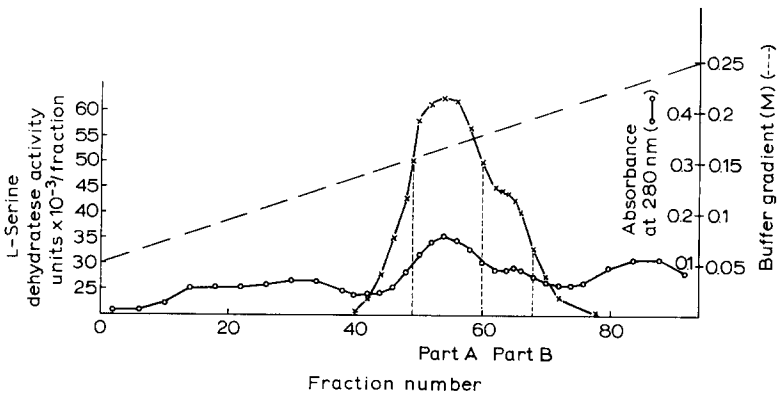

Fig. 1. Elution pattern of rat liver L-serine dehydratase from a DEAE-cellulose column. The conditions are described in the text.

was renewed every 2 months, the enzyme lost only 20% of its activity in one year. The purified enzyme preparation showed a single protein band on polyacrylamide gel electrophoresis as well as on sodium dodecyl sulfate gel electrophoresis. The purification procedures are summarized in Table I.

TABLE I

SUMMARY OF PURIFICATION OF L-SERINE DEHYDRATASE FROM RAT LIVER

Treatment	*Volume (ml)*	*Total protein (mg)*	*Total activity (units × 10^{-3})*	*Specific activity (units/mg protein)*	*Yield (%)*
Crude extract	1790	67 000	2180	32.6	100
First $(NH_4)_2SO_4$ (28–45%)	195	17 300	1880	108.7	86.3
Heat treatment	670	6 000	1760	293	80.8
Second $(NH_4)_2SO_4$ (0–43%)	46	4 770	1750	367	80.4
Sephadex G-100 filtration	368	159	660	4 150	30.3
DEAE-cellulose + ultrafiltration	2.5	17.4	190	16 700	13.3

Studies on the isoenzyme

As Fig. 1 revealed a shoulder in the activity profile of the enzyme, two parts, A and B, were collected separately, and part of each was submitted to analytical disc electrophoresis (Fig. 2). Part A shows one band only, whereas in Part B two bands can be distinguished in the activity stain. Both of these isoenzymes are also observed on electrophoresis of liver crude extract (Fig. 3). The formazan bands were shown to correspond to enzyme activity by determination of the activity after gel fractionation. Artifacts of the electrophoresis were excluded by re-electrophoresis and by variation of the pyridoxal 5'-phosphate content of the enzyme solution.

After elution of the isoenzymes from analytical gels of Part B enzyme, an identical mol. wt of 35 000 for both isoenzymes was determined on sodium dodecyl

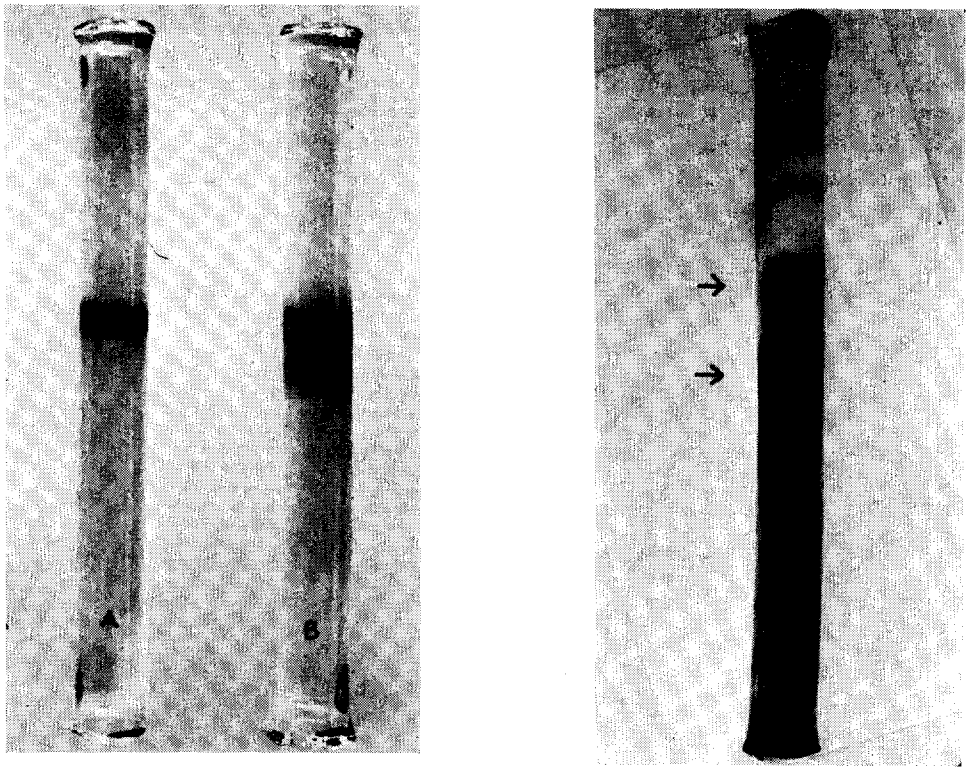

Fig. 2. Activity stain of L-serine dehydratase isoenzymes after polyacrylamide gel electrophoresis. Electrophoresis with 50 μg of enzyme from Part A and 60 μg from Part B after DEAE-cellulose chromatography and staining for activity were carried out as described under Materials and Methods.

Fig. 3. Activity stain of the L-serine dehydratase isoenzymes after electrophoresis of liver crude extract. The livers of rats which had been fed a high protein diet for 7 days were homogenized (1:5) in 5% glucose. Electrophoresis with 0.2 ml of 105 000 $\times$ g supernatant, and staining for activity were performed as described under Materials and Methods. The isoenzymes are indicated by arrows in the figure.

sulfate and sodium dodecyl sulfate–urea gels. A mixture of both enzymes migrated as one band.

Behavior of the enzyme in the presence of the substrate

To see whether the subunit of the native enzyme, composed of two subunits or both are active, we chromatographed 700 units of pure enzyme (Part A) through a Sephadex G-100 column in the presence of the substrate L-serine (0.1 M). The pyridoxal 5'-phosphate concentration ($2 \cdot 10^{-4}$ M) on the column was high enough to prevent inactivation of the enzyme by L-serine, as described by Pestaña and Sols[14]. The enzyme was eluted at a position corresponding to a mol. wt of 60 000. By measuring the pyruvate formed during the gel filtration, the existence of other active forms could be excluded. Molecular weight determinations in the absence of the substrate gave values of 60 000 by gel filtration and 68 000 by sucrose gradient centrifugation.

Inactivation of the enzyme by dilution

At enzyme concentrations lower than 10 μg/ml of the incubation mixture, the enzyme is partially inactivated by dilution. This inactivation can be prevented and partially reversed by K^+ and NH_4^+ (ref. 15). Using Sephadex G-100 filtration, it can be shown that the enzyme dissociates into less active or inactive subunits upon dilution (Fig. 4). The elution volumes on Sephadex G-100 varied from 66 ml for 5 units, 64 ml for 50 units to 56 ml for 1000 units, corresponding to mol. wts of 37 000,

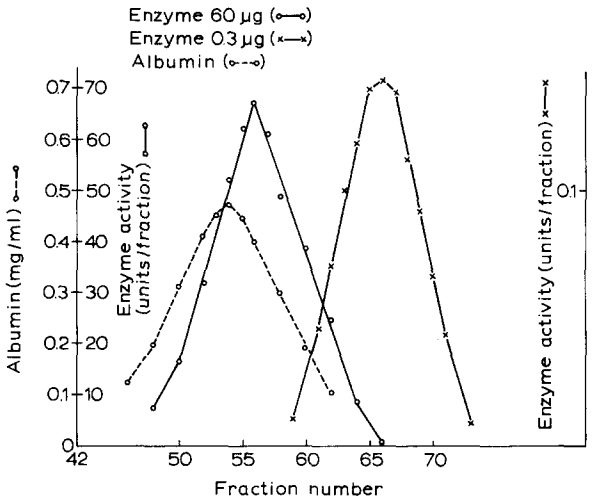

Fig. 4. Sephadex G-100 filtration at different concentrations of L-serine dehydratase. A Sephadex G-100 column (2.5 cm × 24 cm) was equilibrated with 0.1 M sodium pyrophosphate buffer, pH 8.4, containing $2 \cdot 10^{-4}$ M pyridoxal 5'-phosphate. After calibration with standard proteins, 60 μg and 0.3 μg, respectively, of L-serine dehydratase were applied on the column in 2.5 ml of the same buffer and eluted at 25 °C at a constant flow rate of 25 ml/h. 1-ml fractions were collected and assayed for L-serine dehydratase activity in the presence of 0.1 M KCl.

40 000, and 60 000. Similar results were obtained by sucrose gradient centrifugation. By the addition of K^+ or NH_4^+ the equilibrium between the subunit and the holoenzyme can be shifted considerably towards the holoenzyme. Fig. 5 shows that, in the presence of 0.1 M K^+, highly diluted enzyme (50 units) has almost the same elution volume as concentrated enzyme (1000 units).

DISCUSSION

There has been some disagreement about the molecular weight of L-serine dehydratase from rat liver in the past. The following mol. wts have been reported: 64 000 by Nakagawa *et al.*[5], 34 000 for the smallest subunit by Inoue *et al.*[3], 20 000 by Nagabushanam and Greenberg[1], and 42 000 by Hsohino and Kröger[4]. The reason for these differences could have been differences in the purification methods or in the stage of purity.

We purified L-serine dehydratase by a different method to a similar degree of purity as judged from electrophoresis and specific activity, and with a similar recovery as obtained by Nakagawa *et al.*[5]. With this enzyme preparation we obtained

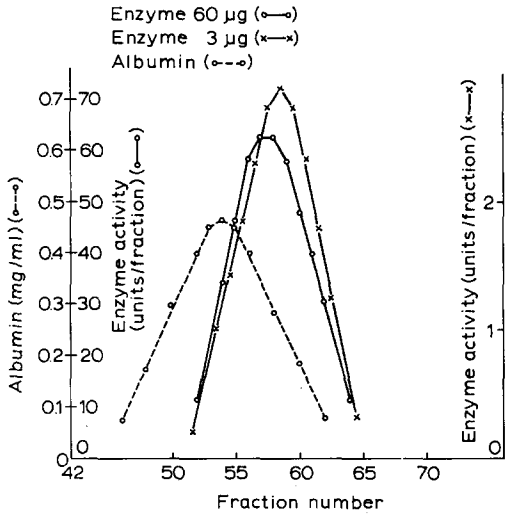

Fig. 5. Sephadex G-100 filtration at different enzyme concentrations in the presence of K^+. 60 µg and 3 µg of enzyme were used. Conditions were the same as in Fig. 4, except that the buffer was 0.1 M in KCl.

mol. wts of 60 000 by Sephadex gel filtration, 68 000 on sucrose gradients, and 35 000 for the smallest subunit by sodium dodecyl sulfate gel electrophoresis. These results confirm the values of Nakagawa *et al.*[5] and Inoue *et al.*[3].

After the last purification step we can distinguish two L-serine dehydratase activities in the DEAE-cellulose elution profile. They are separable by analytical disc electrophoresis, but have identical molecular weights as judged from sodium dodecyl sulfate gel electrophoresis. The two activities can be demonstrated by electrophoresis of the rat liver crude extract, too. This is in good agreement with the findings of Inoue *et al.*[3].

Concerning the subunit structure of the enzyme, gel filtration with 700 units of enzyme in the presence of the substrate L-serine shows the native enzyme, composed of two subunits, as the only active form. We further present some evidence that the process which underlies the inactivation of the enzyme by dilution is the dissociation into subunits, being inactive or much less active than the holoenzyme. K^+ and NH_4^+ can be shown to effectively counteract the dissociation by dilution. They help to reassemble the subunits to the native enzyme, and this should be the way, in which they activate the enzyme at low enzyme concentrations[15]. Moreover, K^+ and NH_4^+ are known to increase the pyridoxal 5'-phosphate–enzyme binding constant[15,16]. They probably act by inducing a slight conformational change in the subunits of the enzyme.

ACKNOWLEDGEMENTS

We thank Miss Petra Schulte for excellent technical assistance. This work was supported in part by grants from the Deutsche Forschungsgemeinschaft and Fond der Chemie.

REFERENCES

1 Nagabushanam, A. and Greenberg, D. M. (1965) *J. Biol. Chem.* 240, 3002–3008
2 Nakagawa, H. and Kimura, H. (1969) *J. Biol. Chem.* 66, 669–683
3 Inoue, H., Kasper, C. B. and Pitot, H. C. (1971) *J. Biol. Chem.* 246, 2626–2632
4 Hoshino, J. and Kröger, H. (1969) *Hoppe-Seyler's Z. Physiol. Chem.* 350, 595–602
5 Nakagawa, H., Kimura, H. and Miura, S. (1967) *Biochem. Biophys. Res. Commun.* 28, 359–364
6 Hoshino, J., Simon, D. and Kröger, H. (1971) *Biochem. Biophys. Res. Commun.* 44, 872–878
7 Davis, B. J. (1964) *Ann. N.Y. Acad. Sci.* 121, 404–427
8 Feldberg, R. S. and Datta, P. (1970) *Science* 170, 1414–1416
9 Ward, S., Wilson, D. L. and Gilliam, J. J. (1970) *Anal. Biochem.* 38, 90–97
10 Weber, K. and Osborn, M. (1969) *J. Biol. Chem.* 244, 4406–4412
11 Andrews, P. (1964) *Biochem. J.* 91, 222–233
12 Martin, R. G. and Ames, B. N. (1961) *J. Biol. Chem.* 236, 1372–1379
13 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* 193, 265–275
14 Pestaña, A. and Sols, A. (1970) *FEBS Lett.* 7, 29–31
15 Hoshino, J., Simon, D. and Kröger, H. (1972) *Eur. J. Biochem.* 27, 388–394
16 Pestaña, A. (1971) *FEBS Lett.* 13, 59–61