Affinity-chromatographic purification of *S*-adenosyl-L-homocysteine hydrolase

Some properties of the enzyme from rat liver

E. Olavi KAJANDER and Aarne M. RAINA

Department of Biochemistry, University of Kuopio, Kuopio, Finland

(Received 8 July 1980/Accepted 8 October 1980)

S-Adenosyl-L-homocysteine hydrolase has been purified to apparent homogeneity from rat liver by means of affinity chromatography on 8-(3-aminopropylamino)adenosine linked to Sepharose. The purified enzyme was free from adenosine kinase and adenosine deaminase activities and was homogeneous on SDS/polyacrylamide-gel electrophoresis, which gave a subunit mol.wt. of 47000. The native enzyme showed some microheterogeneity on polyacrylamide-gel electrophoresis under increased-resolution conditions but was homogeneous on isoelectric focusing (pI 5.6). The molecular weight of the native enzyme was about 220000 as judged by pore-gradient electrophoresis. The native enzyme bound adenosine tightly and showed K_m values of 0.6 μ M, 0.9 μ M and 60 μ M for adenosine, *S*-adenosyl-L-homocysteine and L-homocysteine respectively. The enzyme was rapidly inactivated when incubated in the presence of adenosine, *S*-adenosyl-L-homocysteine or several adenosine derivatives or analogues. Inactivation took place both at 0 and 37°C. Freezing in the absence of glycerol resulted in the appearance of dissociation products of the oligomeric protein. Multimer formation was observed at low thiol concentrations.

S-Adenosylhomocysteine hydrolase catalyses the reversible hydrolysis of Ado-Hcy to adenosine and homocysteine *in vivo* (de la Haba & Cantoni, 1959; Cortese *et al.*, 1974). The opposite reaction is strongly favoured *in vitro* because the equilibrium constant of the reaction lies far in the direction of synthesis. No other route is known for the degradation of Ado-Hcy under physiological conditions in mammals. Ado-Hcy is produced in biological transmethylation reactions utilizing *S*-adenosylmethionine as the methyl donor.

Ado-Hcy is a potent inhibitor of many methyltransferases. These participate in several vital processes, including modifications of DNA, RNA and protein and the synthesis of many small molecules such as neurotransmitters and creatine (for references, see Cantoni, 1977). Owing to the unfavourable equilibrium constant of Ado-Hcy degradation, an increase in the concentration of adenosine and homocysteine may result in the

intracellular accumulation of Ado-Hcy. We have previously demonstrated that, in pyridoxine deficiency, which causes a block in homocysteine catabolism, the concentration of Ado-Hcy in rat liver was markedly elevated (Eloranta *et al.*, 1976). More recently Kredich and co-workers (Kredich & Martin, 1977; Kredich & Hershfield, 1979) showed that elevated adenosine and homocysteine concentrations raised Ado-Hcy concentration in lymphoid cells. These authors suggested that the increase in Ado-Hcy concentration caused cytotoxic effects and that this may have a role in the pathogenesis of severe combined immunodeficiency. Pike *et al.* (1978) showed that *S*-adenosylmethionine-mediated methylation was needed for human monocyte chemotaxis, and elevation of the intracellular Ado-Hcy concentration inhibited this reaction. It therefore appears that factors which cause accumulation of Ado-Hcy may seriously impair important cellular functions.

We have previously described a rapid and sensitive method for the assay of *S*-adenosylhomocysteine hydrolase activity (Kajander *et al.*, 1976). We also noticed that some of the kinetic properties of the enzyme from rat liver differed considerably

Abbreviations used: Ado-Sepharose, 8-(3-aminopropylamino)adenosine linked to CH-Sepharose 4B; Ado-Hcy, *S*-adenosyl-L-homocysteine; SDS, sodium dodecyl sulphate.

from those reported previously. Purification and a closer characterization of this enzyme seemed therefore desirable. In the present paper we describe a simple and rapid purification method for *S*-adenosylhomocysteine hydrolase. By using this method we have isolated the enzyme in a homogeneous form. Some important aspects in respect to avoiding inactivation of the enzyme are also described. In the course of the present work, isolation of *S*-adenosylhomocysteine hydrolase from bovine liver, rat brain, and mouse and rat liver has been reported (Richards *et al.*, 1978; Schatz *et al.*, 1979; Saebø & Ueland, 1978; Chabannes *et al.*, 1979).

Experimental

Materials

The following chemicals were supplied by Sigma Chemical Co., St. Louis, MO, U.S.A.: Ado-Hcy, L-homocysteine thiolactone, 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide, Coomassie Brilliant Blue G, crystalline bovine serum albumin, 8-bromo-adenosine and adenine 9- β -D-arabinofuranoside (arabinosyladenine). L-Homocysteine was prepared from L-homocysteine thiolactone by the method of Duerre & Miller (1966). [8- 14 C]Adenosine (sp. radioactivity 49 mCi/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Radioactive Ado-Hcy was prepared enzymically by the procedure described previously (Eloranta *et al.*, 1976). *erythro*-9-(2-Hydroxy-3-nonyl)adenine was supplied by Burroughs Wellcome Co., Research Triangle Park NC, U.S.A. Sephadex G-200, CH-Sepharose 4B, gel-filtration calibration kit, gel-electrophoresis calibration kit, polyacrylamide-gradient-gel plates 4/30, Sephadex G-10 and PD 10 Sephadex G-25 M columns were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Ampholine polyacrylamide-gel plates were from LKB Instruments, Stockholm, Sweden. Combithek calibration kit for SDS/polyacrylamide-gel electrophoresis and adenosine deaminase from calf intestine were from Boehringer, Mannheim, Germany. Minicon B15 concentrators were from Amicon Corp., Lexington, MA, U.S.A. All other chemicals used were Reagent-Grade materials from various commercial sources.

Methods

Assay of *S*-adenosylhomocysteine hydrolase activity. *S*-Adenosylhomocysteine hydrolase (EC 3.3.1.1) activity was assayed in the direction of Ado-Hcy synthesis as described previously (Kajander *et al.*, 1976). In experiments with purified enzyme, bovine serum albumin (0.5 mg/ml) was added. The radioactive product was separated by paper electrophoresis. For rapid activity measurements, Ado-Hcy was separated from the HClO₄

supernatant by Whatman phosphocellulose ion-exchange paper P81 as described previously (Kajander *et al.*, 1976). However, the papers were washed with 300 ml of a solution containing 1 mM-HCl and 2 mM-KCl instead of 1 mM-HCl. Addition of KCl completely removed radioactive adenosine.

Hydrolysis of Ado-Hcy to adenosine and homocysteine was assayed using radioactive Ado-Hcy (labelled in the adenosine portion) as the substrate. The adenosine deaminase reaction was coupled to the assay. The radioactive inosine formed was separated by paper electrophoresis as described above.

Enzyme activity is expressed as nmol of Ado-Hcy formed/min per mg of protein. One unit of enzyme activity is equal to 1 μ mol of Ado-Hcy formed/min.

Other enzyme assays. Adenosine deaminase (EC 3.5.4.4) activity was assayed by measuring the conversion of [8- 14 C]adenosine into inosine as described by Pull & McIlwain (1974), except that the pH of the phosphate buffer was 7.4. The reaction products (hypoxanthine is also formed with the crude extract as the enzyme) were separated by paper electrophoresis as described above.

Adenosine kinase (EC 2.7.1.20) activity was measured essentially as described by Schnebli *et al.* (1967). The reaction products were separated by paper electrophoresis in NaOH/glycine buffer, pH 11.0, using unlabelled AMP as a marker. In preparations containing adenosine deaminase activity, adenosine kinase was assayed in the presence of 1 μ M-*erythro*-9-(2-hydroxy-3-nonyl)adenine.

Protein determination. Protein was measured by the method of Lowry *et al.* (1951) or with Coomassie Brilliant Blue as described by Spector (1978). Crystalline bovine serum albumin (Sigma) was used as a standard. Both assay methods gave comparable results with crude preparations, but with the purified enzyme the Lowry method gave about 40% lower protein content. In the present paper the protein contents given for the affinity-chromatographic elution and for the purified enzyme refer to the Coomassie Brilliant Blue method.

Disc-gel electrophoresis. Polyacrylamide-gel electrophoresis was performed essentially as described by Davis (1964), with 7% (w/v) acrylamide separating gel (5.5 cm long) in Tris/HCl buffer, pH 8.9, and 2.5% acrylamide spacer gel (1 cm) in Tris/HCl buffer, pH 6.7. The method of electrophoresis and the staining and destaining procedures have been described previously in detail (Pajula *et al.*, 1979). For localization of adenosine-binding activity the purified enzyme was preincubated at 37°C with 1 μ M-adenosine (sp. radioactivity 49 mCi/mmol) for 20 min before polyacrylamide-gel electrophoresis. After the run, the gel was sliced longitudinally and one half was fixed and stained. The

other half was cut into 1 mm sections and each of these was eluted for 24 h with 100 μ l of 0.1 M-potassium phosphate buffer, pH 7.4, containing 2 mM-dithiothreitol. Radioactivity in the eluate was counted in Bray's (1960) solution. A similar method was used for localization of the enzyme activity in the gel.

SDS/polyacrylamide-gel electrophoresis was performed at room temperature, with 7.5% (w/v)- and 10.5%-acrylamide separating gels (6.5 cm long) without spacer gel. Electrophoresis was performed and the gels were stained as described previously (Pajula *et al.*, 1979).

Polyacrylamide-gradient-gel electrophoresis. Gradient-gel electrophoresis was performed by using PAA 4/30 gradient gels (Pharmacia) or gels prepared as described by Rchel & Gross (1979). The gradients were formed with a Beckman density-gradient former (Beckman Instruments, Palo Alto, CA, U.S.A.) instead of Rchel & Gross's (1979) tilt technique. A 5–16% linear gradient slab gel was prepared from ice-cold high- and low-density mixtures (see Rchel & Gross, 1979). The gradient was delivered to a glass cassette (outer dimensions 82 mm $\times$ 82 mm $\times$ 4.9 mm; Pharmacia). Electrophoresis was performed in a Pharmacia electrophoresis apparatus GE-4 according to the manufacturer's instructions, with 90 mM-Tris/80 mM-borate/3 mM-EDTA/2 mM-2-mercaptoethanol/1 mM-dithiothreitol, pH 8.4, for 10–20 h at 125 V. SDS/polyacrylamide-slab-gel electrophoresis was performed in 40 mM-Tris/20 mM-sodium acetate buffer, pH 7.4, containing 2 mM-EDTA, 0.2% SDS, 5 mM-2-mercaptoethanol and 1 mM-dithiothreitol.

Molecular weight was estimated from the calibration curve obtained from the R_m values for the standard proteins or their subunits.

Isoelectric focusing. Isoelectric focusing was performed in an LKB 2117 Multiphor apparatus, with Ampholine/polyacrylamide-gel plates with pH range 4.0–6.5, according to the instructions of the manufacturer. The pH gradient was determined from the surface of the gel by surface electrode (Ingold, Zrich, Switzerland).

Estimation of molecular weight. Gel filtration was performed at 4C on a column (2.6 cm $\times$ 60 cm) of Sephadex G-200 equilibrated with 50 mM-Tris/HCl buffer, pH 7.0, containing 0.1 M-KCl and 1 mM-dithiothreitol. The molecular weight of *S*-adenosyl-homocysteine hydrolase was estimated from the calibration curve obtained from the K_{av} values of the standard proteins (albumin, aldolase, catalase and ferritin).

Results

Preparation of biospecific affinity ligand

In our previous paper (Kajander *et al.*, 1976) we reported that *S*-adenosylhomocysteine hydrolase

has a very high affinity for adenosine. After preliminary experiments the following procedure was adopted. 8-Bromoadenosine was used as a starting material to couple 1,3-diaminopropane to give 8-(3-aminopropylamino)adenosine, which was then linked to CH-Sepharose 4B.

8-(3-Aminopropylamino)adenosine was prepared by nucleophilic displacement of halogen by 1,3-diaminopropane. A mixture of 0.5 mmol of 8-bromoadenosine, 12.5 mmol of 1,3-diaminopropane and 200 μ l of water was heated in a glass-stoppered tube at 99C (see Trayer *et al.*, 1974), with magnetic stirring. During the reaction a shift of the u.v. peak from 265 nm to 279 nm was noted. This shift is characteristic of 8-aminoalkylation of the adenine nucleus (Muneyama *et al.*, 1971). The reaction proceeded essentially to completion in 5 h. After cooling, the liquid was evaporated several times (after addition of water) in a vacuum evapor-

Fig. 1. Purification of 8-(3-aminopropylamino)adenosine by chromatography on Sephadex G-10

A portion of neutralized reaction mixture corresponding to 70 μ mol of the original substrate (8-bromoadenosine) was applied to a column (1.5 cm $\times$ 88 cm) of Sephadex G-10 and eluted with 10 mM-(NH₄)₂CO₃. Fractions 46–69 were pooled and evaporated to dryness. The arrow A shows the void volume of the column (48 ml), B the elution position of diaminopropane and C the elution of a compound tentatively identified as 8-(3-aminopropylamino)adenine.

ator to remove most of the unchanged 1,3-diaminopropane.

Isolation and purification of 8-(3-aminopropylamino)adenosine was performed by adsorption chromatography on a Sephadex G-10 column (see Wasternack & Reinbothe, 1972). This method was especially suitable because salts and compounds lacking an aromatic ring are eluted practically unretarded through the column.

For Sephadex G-10 chromatography the viscous residue left after evaporation was dissolved in water and the pH adjusted to 9.0. A portion corresponding to 70 μ mol of the original substrate was applied to a Sephadex G-10 column (1.5 cm $\times$ 88 cm) equilibrated with 10 mM-(NH₄)₂CO₃ buffer, pH 9.0. The column was eluted with the same buffer at room temperature and 3 ml fractions were collected. The elution profile is shown in Fig. 1. Unchanged 1,3-diaminopropane was eluted soon after the void volume (Fig. 1). The product, 8-(3-aminopropylamino)adenosine, was eluted as a sharp peak, followed by an impurity, probably an adenine derivative. Because there were substantial differences in the elution volumes between the product and impurities, it was possible to increase the sample load up to 300 μ mol. Fractions containing the product were pooled and evaporated to dryness at 55°C in a vacuum evaporator. Evaporation was repeated several times (by adding water) to remove (NH₄)₂CO₃ completely. The yield was 87%.

8-(3-Aminopropylamino)adenosine had the spectral characteristics of 8-substituted adenosine with an absorption maximum at 279 nm (at pH 1 and 11). It was ninhydrin-positive and migrated as a single band on paper electrophoresis with two buffer systems (0.1 M-citrate/NaOH, pH 4.3; 50 mM-glycine/NaOH, pH 11.0). Staining with ninhydrin (Raina *et al.*, 1967) showed the presence of one reactive amino group per adenosine derivative. The molecular weight determined by mass spectrometry (Jeol JMS D-300) was 339, corresponding to the expected value.

The adenosine derivative was coupled to CH-Sepharose 4B by using the carbodi-imide method. The washed gel (8 ml, 2 g dry wt.) was mixed with 300 μ mol of adenosine derivative in 5 ml of water. The mixture was stirred slowly and 300 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide as a fine powder was added slowly. Thereafter the instructions of the manufacturer (Pharmacia) were followed. About 7–9 μ mol of the adenosine derivative was found to be bound/1 ml of gel. The Ado-Sepharose was found to be stable. In a column used several times within a year there was less than 20% decrease in the ligand concentration. It is also noteworthy that the free adenosine derivative was not deaminated by adenosine deaminase from calf intestine.

Purification of S-adenosylhomocysteine hydrolase

Livers of 25 female Wistar rats weighing 140–150 g were rapidly removed under ether anaesthesia, cooled in ice and homogenized with a Potter–Elvehjem homogenizer in 2 vol. of ice-cold 10 mM-potassium phosphate buffer, pH 7.4, containing 1 mM-dithiothreitol. All subsequent operations were performed at 0–4°C. The homogenate was centrifuged first at 12 000 g_{av} and then at 100 000 g_{av} at 4°C for 1 h. The supernatant (129 ml) was fractionated by adding solid (NH₄)₂SO₄, and the proteins that precipitated between 38 and 55% saturation were collected by centrifugation at 14 000 g for 30 min and stored at –80°C until used.

The (NH₄)₂SO₄ fraction was thoroughly dialysed against 20 mM-potassium phosphate buffer, pH 7.4, containing 1 mM-dithiothreitol. Any precipitate formed was removed by centrifugation. The dialysed (NH₄)₂SO₄ fraction (36 ml) was applied to a column (8.5 cm $\times$ 30 cm) of DEAE-cellulose (Whatman DE 52) equilibrated with the dialysis buffer. The column was washed with 4 litres of the same buffer and connected to a linear gradient of 0–200 mM-KCl in the same buffer (total gradient volume 4 litres). Fractions of 15 ml were collected. *S*-Adenosylhomocysteine hydrolase activity was eluted between 5 and 25 mM-KCl. The active fractions (400 ml) were pooled, and the enzyme was concentrated by (NH₄)₂SO₄ precipitation (0–70% saturation).

The precipitate was dissolved in 40 ml of 0.2 M-KCl/50 mM-Tris/HCl, pH 7.0, containing 1 mM-2-mercaptoethanol and 1 mM-dithiothreitol, and dialysed overnight against 200 vol. of the same solution. A 10.5 ml portion of the enzyme preparation was applied to the Ado-Sepharose column (1.5 cm $\times$ 4.3 cm), equilibrated with dialysis buffer. Fractions (3.5 ml) were collected. The column was first eluted with 80 ml of the dialysis solution, followed by 80 ml of the dialysis solution containing 0.4 M-KCl, at a flow rate of 10 ml/h. About 90% of protein, but practically no *S*-adenosylhomocysteine hydrolase activity, was removed at this step. The enzyme was eluted with 10 ml of the dialysis solution containing 0.4 M-KCl and 0.2 mM-Ado-Hcy (or adenosine; see below) at a flow rate of 20 ml/h. Fractions (2.5 ml) were collected. A typical elution pattern of protein and *S*-adenosylhomocysteine hydrolase activity is shown in Fig. 2.

As discussed below, in the presence of adenosine or Ado-Hcy, the enzyme is slowly inactivated. Therefore the fractions obtained by the biospecific elution were applied immediately to PD 10 Sephadex G-25 M columns to remove Ado-Hcy. The columns were eluted with 50 mM-Tris/HCl buffer, pH 7.0, containing 2 mM-dithiothreitol and 10% glycerol. The first 3.5 ml of eluate was collected. Fractions containing the hydrolase activity were pooled, concentrated in a Minicon B15 concentrator, added

Fig. 2. Elution profiles of protein and *S*-adenosylhomocysteine hydrolase activity from chromatography on an *Ado*-Sepharose column

Dialysed enzyme preparation (12.3 mg of protein) obtained after DEAE-cellulose chromatography was applied to an *Ado*-Sepharose column (1.5 cm × 4.3 cm). After the column was washed with the dialysis buffer, the concentration of KCl was raised to 0.4 M (arrow A) and 0.2 mM-adenosine was added (arrow B). Fractions (1.7 ml) were collected. —, Protein concentration; ----, enzyme activity.

with glycerol (final concn. 20%, v/v) and stored at -80°C .

Table 1 summarizes the purification procedure. The final preparation had a specific activity 135-fold that of the crude supernatant. The overall yield was about 40%.

Stability of S-adenosylhomocysteine hydrolase

During the development of the purification procedure for *S*-adenosylhomocysteine hydrolase, we found that the enzyme was inactivated in the presence of adenosine. A similar observation was reported by Hershfield (1979), who found that 2'-deoxyadenosine and arabinosyladenine, and to a lesser extent adenosine, are detrimental to *S*-adenosylhomocysteine hydrolase activity of human placental and lymphoblast extracts. As shown in Table 2, incubation of the purified enzyme at 0°C in the presence of 0.2 or 2 mM-adenosine caused about 35 and 85% loss of the enzyme activity in 3 and 24 h respectively. Inactivation seemed to be practically irreversible, as the enzyme activity could not be restored by extensive dialysis (results not shown). *Ado*-Hcy also caused loss of enzyme

Table 1. Purification of *S*-adenosylhomocysteine hydrolase from rat liver
The crude extract was prepared from 133 g of rat liver and processed as described in the text.

Purification step	Total protein (mg)	Specific activity (units/mg)	Total activity (units)	Yield (%)	Purification factor (fold)
Crude extract	5827	0.044	259	100	1
(NH ₄) ₂ SO ₄	2412	0.085	205	79	2
DEAE-cellulose	181	0.649	118	46	15
<i>Ado</i> -Sepharose	17.3	5.950	103	40	135

Table 2. Influence of adenosine and adenosine derivatives on the stability of *S*-adenosylhomocysteine hydrolase
Purified enzyme was preincubated for 0–72 h at 0°C in 100 mM-potassium phosphate buffer, pH 7.4, containing 5 mM-dithiothreitol, with additions indicated in the Table. The activity of the unincubated enzyme was taken as 100%.

Addition	Concentration (mM)	Time (h)	Hydrolase activity remaining after preincubation (%)				
			0	3	24	48	72
None	—	...	100	104	100	98	98
8-(3-aminopropylamino)adenosine	2.0		93	94	83	70	58
<i>Ado</i> -Hcy	0.2		102	87	45	25	15
	2.0		98	86	55	38	27
Adenosine	0.2*		98	65	14	8	5
	2.0*		102	66	15	9	6

* Correction has been made for the dilution of the radioactive adenosine in the enzyme assay.

activity, but the rate of inactivation was much slower than in the presence of adenosine. About half of the activity was left after incubation for 24 h (Table 2). Interestingly, the inactivation was slower at higher concentrations of Ado-Hcy. As adenosine was more deleterious to the enzyme than was Ado-Hcy, the latter was chosen for the elution of the enzyme from the affinity column. During the biospecific elution, the enzyme was exposed to Ado-Hcy (0.2 mM) for about 1 h. If Ado-Hcy was removed from the enzyme preparation immediately after elution, the loss of enzyme activity was negligible.

Adenosine and Ado-Hcy were equally effective in eluting *S*-adenosylhomocysteine hydrolase from the Ado-Sepharose column, whereas L-homocysteine was totally ineffective. This means that 8-(3-aminopropylamino)adenosine, adenosine and Ado-Hcy probably compete for the same binding site on the enzyme. 8-(3-Aminopropylamino)adenosine was inactive as a substrate in the synthetic reaction catalysed by the enzyme, although it weakly inhibited the synthesis of Ado-Hcy from adenosine and L-homocysteine. A 26% decrease of Ado-Hcy synthesis was observed when 8-(3-aminopropylamino)adenosine was present in a 10^3 -fold excess over adenosine (results not shown). The free adenosine derivative only slowly inactivated the purified hydrolase when incubated at 0°C (Table 2).

In the absence of nucleosides or nucleoside derivatives, the purified enzyme was stable when stored at 0°C in 100 mM-potassium phosphate containing 5 mM-dithiothreitol (Table 2). In the presence of 1 mM-dithiothreitol, the enzyme was stable at 0°C for about 1 month, losing its activity thereafter fairly rapidly (results not shown). The activity could be restored by adding dithiothreitol, indicating that thiol groups are essential for the enzyme activity. Even in the presence of the thiol compound, the enzyme was inactivated by repeated freezing and thawing. Inactivation was prevented by adding glycerol to a 20% (v/v) final concentration. In the presence of glycerol, no loss of activity was detected when the enzyme was stored for 8 months at -80°C.

Properties of the purified enzyme

The purified hydrolase was found to be free of adenosine deaminase and adenosine kinase activity. The last-mentioned enzyme has a high affinity for adenosine (K_m 0.5 μ M; Arch & Newsholme, 1978) and therefore might have been retained on the Ado-Sepharose column. Both enzyme activities were completely eliminated at the DEAE-cellulose-chromatography step. It was further demonstrated (results not shown) that adenosine deaminase was not retained by the Ado-Sepharose column.

The purified enzyme when subjected to analytical-gel electrophoresis on 7%-polyacrylamide gels

Fig. 3. Mobility of native *S*-adenosylhomocysteine hydrolase on polyacrylamide-gel electrophoresis

(a) A sample (5 μ g) of purified enzyme was subjected to electrophoresis in 7%-polyacrylamide gel and the run was continued for 2 h after the tracking dye had migrated out of the gel (total running time 190 min). (b) Samples (1.7 and 3 μ g) of purified enzyme, eluted from Ado-Sepharose column either by 1 M-KCl (nos. 1 and 2) or by 0.2 mM-Ado-Hcy (3 and 4) were subjected to 5–16%-polyacrylamide-gradient-gel electrophoresis for 20 h at 125 V, together with the marker proteins (A–D): A, thyroglobulin from pig thyroid (mol.wt. 669 000); B, ferritin from horse spleen (440 000); C, catalase from ox liver (232 000); D, lactate dehydrogenase from ox heart (140 000).

migrated as a single band under ordinary conditions. The adenosine-binding activity co-migrated with the *S*-adenosylhomocysteine hydrolase activity, which indicates a tight binding of the substrate to the enzyme protein. When the native enzyme preparation was subjected to a prolonged run (for more than 1 h) in polyacrylamide gel, three separate bands gradually appeared (Fig. 3a), indicating some microheterogeneity. The result (not shown) was the same if the enzyme was preincubated in the presence of substrates or inactivated by incubating it with adenosine, Ado-Hcy, arabinosyladenine or 8-(3-aminopropylamino)adenosine (see above).

Microheterogeneity of the native enzyme was also detected in the gradient-gel electrophoresis. The use of a 5–16% gel gradient invariably resulted in the appearance of two major bands that migrated very closely together (Fig. 3b). This indicates small differences in the molecular size and/or conformation. As shown in Fig. 3(b), the two bands were also detected with enzyme preparations not exposed to Ado-Hcy but eluted with 1M-KCl from the Ado-Sepharose column.

The approximate molecular weight of the purified *S*-adenosylhomocysteine hydrolase was determined by polyacrylamide-gradient-gel electrophoresis and by gel filtration in a calibrated column of Sephadex G-200. The gradient gel of 4–30% acrylamide gave a mol.wt. of 220 000. The molecular weights for the two major bands obtained with the gradient gel of 5–16% acrylamide were 224 000 and 229 000 (Fig. 3b). Gel filtration in a calibrated Sephadex G-200 column (see under 'Methods') gave an approx. mol.wt. of 210 000. This value is in a fairly good agreement with those obtained with the gradient-gel electrophoresis. Calculation of the molecular weight has been made assuming only a small or moderate asymmetry of the enzyme molecule (see Ackers & Steere, 1967; Felgenhauer, 1974).

Polyacrylamide-gel electrophoresis of the purified enzyme in the presence of SDS revealed only one protein band, both in homogeneous and gradient gels. The molecular weight of the subunit obtained in homogeneous gels (Fig. 4a) with two polyacrylamide concentrations (7.5 and 10.5%) was 47 000, whereas a somewhat smaller value (45 000) was calculated from the mobility in the polyacrylamide gradient gel (Fig. 4b).

Isoelectric focusing on Ampholine/polyacrylamide-gel plates with pH range 4.0–6.5 revealed one major band with pI 5.6.

Kinetic properties of the purified enzyme

The reaction rate in the direction of synthesis was linear with time and enzyme concentration until about 80% of the substrate adenosine was consumed. The K_m values were 0.6 μ M for adenosine and 60 μ M for L-homocysteine. The rate of hydrolysis of

Fig. 4. Determination of the subunit molecular weight of *S*-adenosylhomocysteine hydrolase by SDS/polyacrylamide-gel electrophoresis

(a) Samples of the purified enzyme were incubated separately (II, III) or together with marker proteins (I) in 1% SDS/5% 2-mercaptoethanol for 3 min at 90°C and subjected to electrophoresis in 7.5% polyacrylamide gel in sodium phosphate buffer, pH 7.2, containing 0.1% SDS, 2 mM-2-mercaptoethanol and 1 mM-dithiothreitol. The amounts of enzyme protein (band D) were 3, 10 and 20 μ g in I, II and III, respectively. (b) *S*-Adenosylhomocysteine hydrolase (1.5 and 3 μ g) and the mixture of marker proteins were incubated in 2.5% SDS/5% 2-mercaptoethanol for 5 min at 100°C and applied to a SDS/polyacrylamide slab gradient gel (PAA 4/30, Pharmacia) in 40 mM-Tris/20 mM-sodium acetate / 2 mM-EDTA / 2 mM-2-mercaptoethanol / 1 mM-dithiothreitol/0.2% SDS. Electrophoresis was continued for 30 min after the tracking dye had migrated off the gel. The marker proteins in (a) and (b) were as follows: A, β' - and β -subunits of *Escherichia coli* RNA polymerase (the subunit mol.wts. 165 000 and 155 000); B, bovine serum albumin (67 000); C, catalase (60 000); E, α -subunit of RNA polymerase (39 000); F, lactate dehydrogenase (36 000); G, soya-bean trypsin inhibitor (21 500); H, ferritin (18 500).

Fig. 5. Hydrolysis of Ado-Hcy as a function of substrate concentration

The incubation mixture contained 0.5–10 μM -Ado-Hcy, 2 units of adenosine deaminase, 0.5 mg/ml bovine serum albumin and purified *S*-adenosylhomocysteine hydrolase (0.16 μg). The incubation time was 5 min.

Ado-Hcy by the purified enzyme was linear with enzyme concentration, but not with time, even in the presence of added adenosine deaminase. This may at least partly be explained by the observation (Ueland & Saebø, 1979a) that the hydrolase can protect adenosine from being deaminated. Accumulation of homocysteine may also inhibit the reaction. The K_m value for Ado-Hcy in the hydrolytic reaction was 0.9 μM (Fig. 5).

Multimer formation

Guranowski & Pawelkiewicz (1977) have observed a tendency of the purified *S*-adenosylhomocysteine hydrolase from yellow-lupin (*Lupinus luteus*) seeds to form multimers on repeated freeze-thawing. The same appears to be true for the enzyme purified from rat liver. Repeated freezing and thawing in the absence of glycerol inactivated the enzyme, with an appearance of several bands on polyacrylamide-gel electrophoresis, some migrating faster and some more slowly than the native enzyme. Decrease in the thiol concentration (<1 mM-2-mercaptoethanol) and exposure of the enzyme

Fig. 6. Dissociation and aggregate formation of purified *S*-adenosylhomocysteine hydrolase

(a) Purified enzyme (25 μg) in 50 mM-Tris/HCl buffer, pH 7.0, containing 1 mM-2-mercaptoethanol was stored frozen for 1 week, thawed and subjected to electrophoresis in 7% polyacrylamide gel. The Tris/HCl buffer (pH 8.9) used for electrophoresis contained 1 mM-2-mercaptoethanol, but no dithiothreitol. A, Tracking dye; B, subunit of the enzyme; C, active enzyme, possibly a pentamer; D–H, multimers. (b) A plot of the R_m values of the bands as a function of their presumed molecular weights. For details, see the text.

preparation to atmospheric O_2 for several days fortified the aggregate formation. A typical dissociation–aggregation pattern detected by polyacrylamide-gel electrophoresis is shown in Fig. 6(a). Band B probably represents the subunit (i.e. the monomer, mol.wt. 47000), band C the native enzyme (the only band showing *S*-adenosylhomocysteine hydrolase activity) and bands D–H the multimeric forms of the enzyme. The proteins migrating between B and C were probably aggregates (dimers etc.) unstable under the conditions used. In separate experiments with an enzyme partially denatured by dilution, also the formation of dimers, trimers and tetramers was observed (results not shown). In Fig. 6(b) the R_m values (the migration distance of an individual band divided by that of the tracking dye) of the bands were plotted as a function of the subunit molecular weight (47000), assuming that the band C represents a pentamer, band D a hexamer, etc. The result from this plot was a straight line. As a control test, multimer formation of bovine serum albumin (see Sogami & Foster, 1962) was studied under similar conditions. Also in this case a plot of the R_m values against the molecular weights of the monomer to hexamer resulted in a straight line (not shown).

Discussion

The purified enzyme was homogeneous on SDS/polyacrylamide-gel electrophoresis, which gave a subunit mol.wt. of 47000. This value perfectly agrees with those (47000–48000) reported for the hydrolase isolated from rat liver (Chabannes *et al.*, 1979) and brain (Schatz *et al.*, 1979), bovine liver (Palmer & Abeles, 1979) and human placental enzyme (Hershfield & Kredich, 1978). The variation in the molecular weights reported for the native enzyme isolated from various mammalian sources is much larger, ranging from 180000 (rat brain; Schatz *et al.*, 1979) to 220000 (bovine liver; Richards *et al.*, 1978) or even higher. Generally, the lower values were obtained by analytical ultracentrifugation. In the present study the molecular weight obtained for the native enzyme by gel filtration and pore-gradient-gel electrophoresis varied between 210000 and 229000. Although these values as well as the multimer-formation analysis suggest that the native enzyme might be a pentamer, confirmation by other methods is definitely needed. Interestingly, an adenosine-binding protein (probably identical with *S*-adenosylhomocysteine hydrolase) isolated from rabbit erythrocytes was supposed to be a pentameric protein (Yuh & Tao, 1974).

The present results demonstrate that the addition of glycerol and a thiol compound, preferably dithiothreitol, effectively prevented the loss of activity on prolonged storage or by freezing and

thawing. Furthermore, we observed that the enzyme rapidly lost activity when incubated with adenosine, Ado-Hcy and various other nucleoside derivatives. It is noteworthy that inactivation took place at a low temperature (0°C). A 'suicide'-type inactivation of *S*-adenosylhomocysteine hydrolase from human lymphoblasts has been reported to occur when the enzyme was incubated at 37°C in the presence of various nucleosides, arabinosyladenine being especially effective (Hershfield, 1979). We noticed that the inactivation by adenosine was practically irreversible, which suggests an inactivation mechanism similar to that described by Hershfield (1979). This observation has two implications. Firstly, the time of exposure of the enzyme to adenosine, Ado-Hcy or other nucleoside derivatives must be limited to a minimum during affinity-chromatographic purification. This might partly explain the higher specific activity and yield of the purified enzyme obtained in the present study as compared with that reported by Chabannes *et al.* (1979), who also used Ado-Hcy for the elution of the enzyme in the affinity chromatography (6-mercaptopurine 9-D-riboside as the ligand). Secondly, as pointed out by Hershfield (1979), inactivation of *S*-adenosylhomocysteine hydrolase may also occur in cells treated with adenine nucleosides (adenosine, 2'-deoxyadenosine, arabinosyladenine, etc.), especially when combined with an adenosine deaminase inhibitor, thereby contributing to the accumulation of Ado-Hcy. This in turn may interfere with vital cellular reactions such as trans-methylations.

The K_m values, 0.6 μM for adenosine and 60 μM for L-homocysteine, obtained with the purified enzyme in the present study, perfectly agree with those previously reported for the crude enzyme from rat liver (Kajander *et al.*, 1976), and show that the much higher values previously obtained must be erroneous (for references, see Kajander *et al.*, 1976). This conclusion is further strengthened by the recently reported K_m values for adenosine, 0.9 μM for human placental enzyme (Hershfield & Kredich, 1978) and 0.2 μM for *S*-adenosylhomocysteine hydrolase from mouse liver (Ueland & Saebø, 1979b). We obtained a K_m value of 0.9 μM for Ado-Hcy in the hydrolytic reaction, which is close to that (0.75 μM) reported by Ueland & Saebø (1979b). Higher values (37 and 63 μM) were reported for the enzymes isolated from rat brain and bovine liver (Schatz *et al.*, 1979; Richards *et al.*, 1978). The apparent discrepancy between these values may be due to the non-linearity of the hydrolytic reaction with time. The non-linearity of the hydrolytic reaction also makes comparison of the specific activities reported for the purified enzymes difficult. This difficulty is eliminated by measuring the enzyme activity in the direction of synthesis.

Finally, it should be mentioned that the liver is fairly rich in *S*-adenosylhomocysteine hydrolase enzyme protein. From our results one can roughly calculate that it composes about 0.7% of total soluble protein in rat liver. For mouse liver the corresponding value is even higher, i.e. 1.7% (Ueland & Saebø, 1979a). Thus the elimination of Ado-Hcy formed in transmethylation reactions is effective under normal conditions.

This study has been supported by grants from the North Savo Fund of the Finnish Cultural Foundation, the Orion Corp. Research Foundation, the National Research Council for Medical Sciences (Finland) and the Sigrid Jusélius Foundation. We thank Mr. Kim Wikstöm of this University for performing the mass-spectral analysis.

References

- Ackers, G. K. & Steere, R. L. (1967) *Methods Virol.* **2**, 325–365
- Arch, J. R. S. & Newsholme, E. A. (1978) *Biochem. J.* **174**, 965–977
- Bray, G. A. (1960) *Anal. Biochem.* **1**, 279–285
- Cantoni, G. L. (1977) in *The Biochemistry of Adenosylmethionine* (Salvatore, F., Borek, E., Zappia, V., Williams-Ashman, H. G. & Schlenk, F., eds.), pp. 557–577, Columbia University Press, New York
- Chabannes, B., Cronenberger, L. & Pacheco, H. (1979) *Experientia* **35**, 1014–1016
- Cortese, R., Perfetto, E., Arcari, P., Protta, G. & Salvatore, F. (1974) *Int. J. Biochem.* **5**, 535–545
- Davis, B. J. (1964) *Ann. N. Y. Acad. Sci.* **121**, 404–427
- de la Haba, G. & Cantoni, G. L. (1959) *J. Biol. Chem.* **234**, 603–608
- Duerre, J. A. & Miller, C. H. (1966) *Anal. Biochem.* **17**, 310–315
- Eloranta, T. O., Kajander, E. O. & Raina, A. M. (1976) *Biochem. J.* **160**, 287–294
- Felgenhauer, K. (1974) *Hoppe-Seyler's Z. Physiol. Chem.* **355**, 1281–1290
- Guranowski, A. & Pawelkiewicz, J. (1977) *Eur. J. Biochem.* **80**, 517–523
- Hershfield, M. S. (1979) *J. Biol. Chem.* **254**, 22–25
- Hershfield, M. S. & Kredich, N. M. (1978) *Science* **202**, 757–760
- Kajander, O., Eloranta, T. & Raina, A. (1976) *Biochim. Biophys. Acta* **438**, 522–531
- Kredich, N. M. & Hershfield, M. S. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 2450–2454
- Kredich, N. M. & Martin, D. W., Jr. (1977) *Cell* **12**, 931–938
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Muneyama, K., Bauer, R. J., Shuman, D. A., Robins, R. K. & Simon, L. N. (1971) *Biochemistry* **10**, 2390–2395
- Pajula, R.-L., Raina, A. & Eloranta, T. (1979) *Eur. J. Biochem.* **101**, 619–626
- Palmer, J. L. & Abeles, R. H. (1979) *J. Biol. Chem.* **254**, 1217–1226
- Pike, M. C., Kredich, N. M. & Snyderman, R. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 3928–3932
- Pull, I. & McIlwain, H. (1974) *Biochem. J.* **144**, 37–41
- Raina, A., Jansen, M. & Cohen, S. S. (1967) *J. Bacteriol.* **94**, 1684–1696
- Richards, H. H., Chiang, P. K. & Cantoni, G. L. (1978) *J. Biol. Chem.* **253**, 4476–4480
- Rüchel, R. & Gross, J. (1979) *Anal. Biochem.* **92**, 91–98
- Saebø, J. & Ueland, P. M. (1978) *FEBS Lett.* **96**, 125–128
- Schatz, R. A., Vunnam, C. R. & Sellinger, O. Z. (1979) in *Transmethylation: Developments in Neuroscience* (Usdin, E., Borchardt, R. T. & Creveling, C. R., eds.), vol. 5, pp. 143–153, Elsevier/North-Holland, New York
- Schnebli, H. P., Hill, D. L. & Bennett, L. L., Jr. (1967) *J. Biol. Chem.* **242**, 1997–2004
- Sogami, M. & Foster, J. F. (1962) *J. Biol. Chem.* **237**, 2514–2521
- Spector, T. (1978) *Anal. Biochem.* **86**, 142–146
- Trayer, I. P., Trayer, H. R., Small, D. A. P. & Bottomley, R. C. (1974) *Biochem. J.* **139**, 609–623
- Ueland, P. M. & Saebø, J. (1979a) *Biochim. Biophys. Acta* **587**, 341–352
- Ueland, P. M. & Saebø, J. (1979b) *Biochemistry* **18**, 4130–4135
- Wasternack, C. & Reinbothe, H. (1972) *J. Chromatogr.* **73**, 135–150
- Yuh, K.-C. M. & Tao, M. (1974) *Biochemistry* **13**, 5220–5226