S-ADENOSYL-L-HOMOCYSTEINE HYDROLASE FROM MOUSE LEUKEMIC CELLS: ISOLATION AND PROPERTIES*

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S-Adenosyl-L-homocysteine hydrolase from mouse leukemic cells L1210 was purified to evident homogeneity with no concomitant enzymatic activities. The isoelectric point of the enzyme is 5·65 and the optimum temperatures for the synthesis and hydrolysis are 52°C and 47°C, respectively. The respective K_M values for adenosine and S-adenosyl-L-homocysteine (SAH) are 3·8 and 4·4 μmol l^{-1}. The optimum pH value for the synthesis of SAH is 6·95—7·20 and for the hydrolysis 7·15—7·80. The enzyme is competitively inhibited by 9-(*S*)-(2,3-dihydroxypropyl)adenine and inactivated with both enantiomers of eritadenine and 3-(adenin-9-yl)-2-hydroxypropanoic acid.

S-Adenosyl-L-homocysteine hydrolase (E.C. 3.3.1.1; further SAH-hydrolase) catalyzes the reversible hydrolysis of SAH to adenosine and L-homocysteine[1,2]. The role of this key regulatory enzyme, responsible for the SAH catabolism in eukaryotic cells acquires even more significance due to the fact that SAH, which is an efficient inhibitor of S-adenosyl-L-methionine (SAM) mediated methylations, catalyzed by methyltransferases[3,4], cannot be removed from the cells by cell membrane transport[5]. It was shown recently that the inhibition or the inactivation of SAH-hydrolase affects the SAM/SAH ratio in the cell[6]. This mechanism probably accounts for the virostatic[2,7], antimalaric[8] and oncostatic[9,10] effects of SAH-hydrolase inhibitors. Therefore, the investigation of properties of SAH-hydrolases from various sources, together with rational synthesis of their effective inhibitors[2,11,12], represents one of the ways to influence biological methylations[13]. Since these processes are very active in different proliferating systems, it is clear that potent inhibitors of SAH-hydrolase may be of potential practical value.

In this work we describe the isolation and the characterization of SAH-hydrolase from mouse leukemic cells L1210. The enzyme was purified by affinity chromatography combined with chromatofocusing. The affinity chromatography was performed on a column of CH-Sepharose 4 B with an attached aliphatic adenosine analogue ligand (see ref.[14]).

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Washed leukemic cells, suspended in a 0·01 mol l^{-1} phosphate buffer pH 7·4, containing 1·0 mmol l^{-1} dithiothreitol were disrupted by sonication and centrifuged at 30 000 *g*. The resulting supernatant fraction was centrifuged at 100 000 *g* and nucleic acids were precipitated with 15% streptomycin sulfate; after centrifugation the supernatant was fractionated with ammonium sulfate. SAH-hydrolase activity was present in the fraction which precipitated in the range of 20–45% saturation with ammonium sulfate. The cytosol was desalted on a column of Sephadex G-25 (coarse) in 0·01 mol l^{-1} phosphate buffer pH 7·4, containing 1·0 mmol l^{-1} dithiothreitol and subjected to chromatofocusing on a PBE 94 column. SAH-hydrolase which was eluted with sharp maximum at pH 5·65 was distinctly separated from adenosine aminohydrolase (E.C. 3.5.4.4), eluted at pH 4·65 (Fig. 1).

The fractions with SAH-hydrolase activity were combined for further purification by affinity chromatography on a modified Sepharose 4B with an attached ligand I. The binding to the affinity column was performed directly in the polybuffer PB 74 and the contaminating proteins were eluted by increasing ionic strength[14]. SAH-hydrolase was then eluted from the column with 0·25 mmol l^{-1} adenosine in 0·75 mol . l^{-1} KCl, containing 1·0 mmol l^{-1} dithiothreitol and adenosine was removed from the eluate by chromatography on a column of Sephadex G-25 (medium)[15]. The enzyme preparation in 0·01 mol l^{-1} phosphate buffer with 1·0 mmol l^{-1} dithiothreitol was made up with glycerol to 20% final concentration to prevent multimer formation[15]. In this form the enzyme was stored at −70°C for several months without any loss of activity. The specific activity of the final enzyme preparation was 6·8 E.U. mg^{-1} as compared with the activity of 0·038 E.U. mg^{-1} of the 20–45% ammonium sulfate saturation fraction (Table I). Electrophoresis of the purified enzyme in 5% as well as in 7·5% polyacrylamide gel revealed a single band associated with the enzyme activity. The SAH-hydrolase activity and the identity of the reaction products during the whole procedure was followed by HPLC, paper chromatography and electrophoresis. The enzyme shows very steep temperature optima at relatively high temperatures: the optimum for the synthesis of SAH is at 52°C, for the hydrolysis at 47°C. Although the difference amounts to only 5°C, it is significant. At higher temperatures, the enzyme is very rapidly inactivated (Fig. 2). A significant difference was also found between the pH optima, observed between pH 6·95–7·20 for the synthesis and 7·15–7·80 for the hydrolysis; see Fig. 3. The pH optimum for hydrolysis is less steep, particularly in the direction towards higher pH values. Interestingly enough, the enzyme exhibits roughly the same affinity towards both adenosine and SAH (the K_M constant at 37°C and pH 7·4: for adenosine 3·8 μmol l^{-1}, for SAH 4·4 μmol l^{-1} in distinction to SAH-hydrolases from other materials[16–19]). In the course of these measurements a strong substrate inhibition of SAH synthesis by adenosine was confirmed[1].

Recent reports describe a number of potent inhibitors of SAH-hydrolases, based on chemical modification of the adenosine moiety[7,10]. In our previous communica-

tions of this series[20-22] we described two new types of highly active inhibitors with chemically modified sugar moiety of adenosine (natural substrate/inhibitor). These inhibitors comprise both neutral compounds, represented *e.g.* by 9-(*S*)-(2,3-dihydroxypropyl)adenine ((*S*)-DHPA, compound *II*) and related derivatives of carboxylic acid

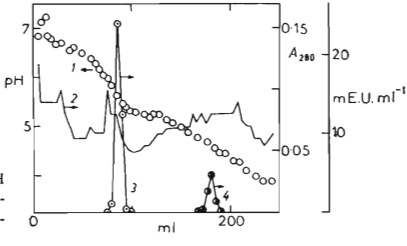


FIG. 1

Chromatofocusing of SAH-hydrolase. *1* pH gradient, *2* absorbance at 280 nm, *3* SAH-hydrolase activity, *4* adenosine amino-hydrolase activity

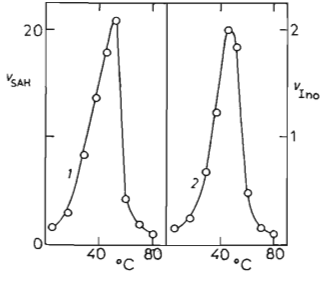


FIG. 2

Optimum temperature for SAH-hydrolase activity. *1* in the direction of SAH synthesis, *2* in the direction of SAH hydrolysis. The reaction rates of synthesis (v_{SAH}) and hydrolysis (v_{Ino}) are given in μmol min^{-1} . mg^{-1}

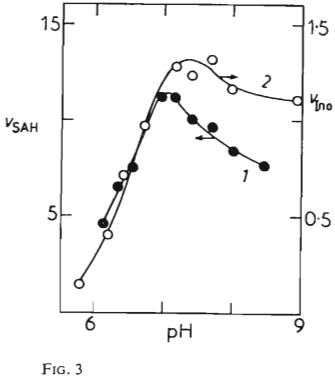
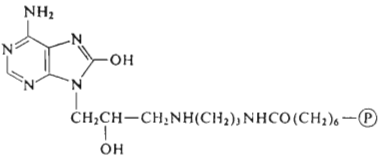


FIG. 3

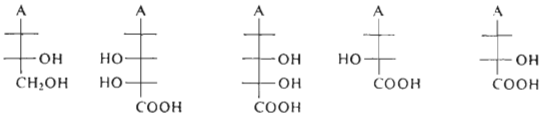
Optimum pH for SAH-hydrolase activity. *1* in the direction of SAH synthesis, *2* in the direction of SAH hydrolysis. The reaction rates of synthesis (v_{SAH}) and hydrolysis (v_{Ino}) are given in μmol min^{-1} mg^{-1}

structure, such as the enantiomeric eritadenines *III* and *IV* or 3-(adenin-9-yl)-2-hydroxypropanoic acids *V* and *VI*. Whereas DHPA (*II*) and other neutral inhibitors exhibit a reversible inhibitory effect[20], the carboxylic acids (*e.g. III* and *IV*) inactivate



I

Ⓟ represents Sepharose 4 B matrix



II (2*S*) | *III* (2*R*,3*R*) | *IV* (2*S*,3*S*) | *V* (2*R*) | *VI* (2*S*)

In formulae *II*–*VI*, A represents adenin-9-yl residue.

the enzyme irreversibly. These properties have been confirmed using SAH-hydrolases from various materials[22]. Within the framework of investigations on SAH-hydrolase from leukemic cells L1210 we studied therefore also the inhibitory effect of two types

TABLE I

SAH-Hydrolase purification (final steps)

Step	Volume ml	Protein mg	Total activity E.U.	Specific activity E.U. mg^{-1}
20–45% Ammonium sulfate	10·0	8·5	0·325	0·038
Chromatofocusing	15·0	—	0·225	—
Affinity chromatography	6·6	0·0092	0·063	6·82

of inhibitors upon this enzyme. Similarly as with rat-liver SAH-hydrolase[11] D-eritadenine proved to be the most active compound which showed a 50% inhibition of SAH hydrolysis at a concentration more than 1 000 times lower than that of the substrate (Table II), and 50% inhibition of SAH synthesis at a concentration 250 times lower than that of adenosine. The inhibition with L-eritadenine is by one order of magnitude lower and the IC_{50} values (Table II) agree with our previous results[22]. It is evident that SAH-hydrolase from L1210 mouse leukemic cells is inhibited by the above-mentioned inhibitors in the same manner as the enzyme isolated from rat liver tissue[22].

The pI value of the SAH-hydrolase from mouse leukemic cells L1210 is similar to the value reported for enzymes isolated from other animal sources[15,17,23–26] whereas the pH optimum values[17,25] differ significantly. The enzyme, which we have isolated by affinity chromatography using the ligand *I*, exhibits a high specific activity of 6·8 E.U. mg^{-1}. Its affinity towards adenosine and SAH is practically the same ($K_M^{Ado} = 3{\cdot}8\ \mu mol\ l^{-1}$, $K_M^{SAH} = 4{\cdot}4\ \mu mol\ l^{-1}$), contrary to the majority of data reporting that the affinity of SAH-hydrolases towards adenosine is higher by one order of magnitude than towards SAH[16–19]. A similarly close affinity towards both of these substrates was observed only with the rat liver enzyme isolated by Finnish authors[15]. Specific activity of their preparation was also substantially higher than that of enzymes described in other papers[1,16,17]. Since their isolation procedure, like that employed by us, involved an effective and rapid enzyme purification by affinity chromatography, it cannot be excluded that isolation techniques which do not use affinity chromatography[1,16,17] afford preparations whose low specific activities are probably due to a partial inactivation resulting in the decreased affinity towards the substrate which, *in vivo* is rather SAH than adenosine[13].

TABLE II

Effect of the inhibitors on SAH-hydrolase catalyzed reactions

Inhibitor	Hydrolysis IC_{50}, $\mu mol\ l^{-1}$ [a]	Synthesis IC_{50}, $\mu mol\ l^{-1}$ [a]
II	0·9	37·0
III	0·003	0·074
IV	0·025	1·7
V	0·04	1·5
VI	0·12	0·46

[a] IC_{50} denotes inhibitor concentration, causing a 50% inhibition of the enzyme after incubation for 10 min.

EXPERIMENTAL

Materials. Leukemic cells L1210 were obtained from female mice DBA/2 (25 g) on the eight day after inoculation with 10^5 cells and they were immediately washed[27]. 9-(2,3-Dihydroxypropyl)adenine (*II*), D-eritadenine (*III*), L-eritadenine (*IV*), (2*R*)-3-(adenin-9-yl)-2-hydroxypropanoic acid (*V*) and (2*S*)-3-(adenin-9-yl)-2-hydroxypropanoic acid (*VI*) were prepared according to ref.[11,12]. Stock solutions of L-homocysteine and [^{14}C]-S-adenosyl-L-homocysteine were prepared as described[20]. Adenosine aminohydrolase (E.C. 3.5.4.4) was purchased from Boehringer (Mannheim, West Germany), Coomassie Brilliant Blue R-250 and G-250 from Fluka (Buchs, Switzerland) and the polybuffer exchanger PBE 94 and polybuffer PB 74 for chromatofocusing from Pharmacia Fine Chemicals (Uppsala, Sweden). [Adenine-U-^{14}C]-adenosine was prepared in the Institute for Research, Production and Application of Radioisotopes (Prague, Czechoslovakia). The affinity column (CH-Sepharose 4B with *I*) was prepared according to ref.[14].

Analysis. The reaction products were analyzed by paper chromatography and radioactivity of the single spots was determined by liquid scintillation or, during the isolation, by HPLC on a 3·3 × 150 mm glass column, packed with Separon SIX C18 5 μm (Laboratory Instruments, Prague, Czechoslovakia); mobile phase: 0·01 mol l^{-1} ammonium dihydrogen phosphate pH 2·8 in 10% methanol; flow rate 0·45 ml min^{-1}, detection at 254 nm, sensitivity 0·08—0·16 absorbancy units per full scale. SAH was identified by paper electrophoresis (22 V/cm, 1 h) on a Whatman No 3 paper (36 × 15 cm) in 1·0 mol l^{-1} acetic acid. Proteins were determined using Coomassie Brilliant Blue G-250 at 607 nm[30].

Enzyme assays. The synthetic activity of SAH-hydrolase was measured in a standard reaction mixture[20,22] (total volume of 0·25 ml), containing 0·05 mol l^{-1}) Sörensen sodium-potassium phosphate buffer pH 7·4 (further only phosphate buffer), 1·6 mmol l^{-1} dithiothreitol, 3·0 mmol l^{-1} L-homocysteine, $2 . 10^{-5}$ mol l^{-1} [^{14}C]-adenosine (specific activity 0·37 MBq μmol^{-1}; for the determination of the K_M values 0·35—10·0 . 10^{-5} mol l^{-1} and 0·028—0·056 μg ml^{-1} of the enzyme preparation. The hydrolytic activity was measured in standard reaction mixture[20,22] (total volume of 0·25 ml), containing 0·05 mol l^{-1} phosphate buffer pH 7·4, 0·1 mmol l^{-1} EDTA, $4 . 10^{-6}$ mol l^{-1} [^{14}C]-SAH (specific activity 1·83 MBq μmol^{-1}); for the determination of the K_M values 0·2—4·0 . 10^{-5} mol l^{-1}), 0·054—0·112 μg ml^{-1} enzyme preparation and 1·6 E.U. ml^{-1} adenosine aminohydrolase. The reaction was started by adding the enzyme preparation and the mixture was incubated at 37°C for 10 min. In the IC_{50} determinations, inhibitors were added to the reaction mixture. For the estimation of the pH dependence of the synthetic reaction, the experiments were performed in 0·09 mol l^{-1} phosphate buffer with $6 . 10^{-4}$ mol l^{-1} L-homocysteine. For SAH-hydrolase assays during chromatofocusing, 20 μl aliquots from each fraction were incubated in the standard reaction mixture. In the determination of the temperature dependence the incubation time was 5 min.

Electrophoresis in polyacrylamide gel. Discontinuous electrophoresis in 5% and 7·5% polyacrylamide gel was performed at a constant current (1·5 mA per sample at 200 to 280 V) and 4°C according to ref.[27,28], using a separation gel with 0·375 mol l^{-1} Tris-HCl buffer pH 8·8 and 2·5% stacking gel with 0·125 mol l^{-1} Tris-HCl buffer pH 6·8. The electrophoresis was carried out in Tris-glycine buffer (0·1 mol l^{-1} glycine, 0·04 mol l^{-1} Tris) pH 8·3. The gels were stained with Coomassie Brillant Blue R-250 or sliced at a thickness of 5 mm; enzymatic activity was determined in a standard reaction mixture at adenosine concentration of $5 . 10^{-6}$ mol l^{-1} and a 20 min incubation period.

Chromatofocusing. Chromatofocusing was carried out at 4°C on a column of Polybuffer Exchanger PBE 94 (0·8 × 40 cm) according to ref.[29,30]. A column, equilibrated with 0·025 mol . . l^{-1} Tris-HCl buffer pH 7·4, was overlayed with polybuffer PB 74 pH 4·0 (5 ml; 1 : 7 dilution

with water) and then 5—10 ml of the sample, containing 5—10 mg of proteins was applied. The elution was carried out with the elution polybuffer at a rate of 35 ml h^{-1}. All the buffers contained 1·0 mmol l^{-1} dithiothreitol.

Affinity chromatography. The affinity gel prewashed with 0·01 mol l^{-1} phosphate buffer pH 7·4, containing 1·0 mmol l^{-1} dithiothreitol, was added directly into the material obtained by chromatofocusing (0·9 ml of the gel per 15 ml of the enzyme preparation). After SAH-hydrolase had been bound, the gel was washed in a 0·8 × 1·8 cm column at 4°C successively with 0·01 mol l^{-1} phosphate buffer pH 7·4 (3 ml), 0·2 mol l^{-1} phosphate buffer pH 7·4 (3 ml), 1·0 mol l^{-1} KCl (3 ml), 1·5 mol l^{-1} KCl (3 ml) and 2·0 mol l^{-1} KCl (5 ml), all containing dithiothreitol (1·0 mmol l^{-1}). SAH-hydrolase was finally eluted with 2·5 . 10^{-4} mol l^{-1} adenosine in 0·75 mol . l^{-1} KCl, containing dithiothreitol (1·0 mmol l^{-1}).

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