AFFINITY CHROMATOGRAPHY OF RAT LIVER S-ADENOSYL-L-HOMOCYSTEINE HYDROLASE*

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A comparison of eight types of carriers for purification of S-adenosyl-L-homocysteine hydrolase by affinity chromatography is described. The Sepharose-based carriers contained stereoisomeric eritadenines bonded by the carboxyl function or neutral aliphatic adenine derivatives bonded by the amino group of substituent on the heterocyclic nucleus or in the side-chain. Materials with bonded isomeric 9-(*RS*)-(3(2)-(3-aminopropylamino)-2(3)-hydroxypropyl)-8-hydroxyadenines (*VIIIb*, *IXb*) appeared to be carriers of choice which enabled an efficient affinity purification of the enzyme from rat liver. SAH-Hydrolase was liberated only upon elutions with dilute adenosine solutions.

S-Adenosyl-L-homocysteine hydrolase (SAH-hydrolase; E.C. 3.3.1.1) represents an important enzyme of the regulatory mechanism of transmethylation reactions in eucaryotic tissues which catalyses the reversible reaction

$$\text{S-adenosyl-L-homocysteine} \rightleftharpoons \text{adenosine} + \text{homocysteine}$$

and is the key enzyme in the S-adenosyl-L-homocysteine (SAH) catabolism in the eucaryotic cells[1]. Since SAH which is necessarily formed in all S-adenosyl-L-methionine-mediated methylations is a strong inhibitor of transmethylases, the removal of excess SAH is a necessary condition for a balanced biosynthesis of proteins or nucleic acids or other metabolic processes. The biological significance of this enzyme is stressed also by the recent finding of its role in the initiation of the virus replication[2]. For these reasons, great attention is paid recently to the isolation of SAH-hydrolases from various sources (mouse[3], rat[4,5], calf[6] and bovine[7] liver, brain tissue[8], lupin seeds[9] or leukemia cells[10]). Since these enzymes are relatively unstable, an application of classical protein purification methods to their isolation from various tissues or cell cultures is not very promising. For this case, specific affinity chromatography seems to be the technique of choice.

Purification of SAH-hydrolase by affinity chromatographic methods has already

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been described: one procedure used a carrier with adenosine, bonded covalently to the polymer matrix by its N^6-amino group[11], the other carrier contained adenosine bonded by the 3-aminopropylamino group in position 8 of the adenine ring[5]. Both these procedures are thus based on interaction with an adenosine analogue which is simultaneously both a substrate and an inhibitor of reactions, catalyzed by the enzyme. We observed recently that some adenosine analogues with an open sugar ring (the so-called aliphatic adenosine analogues) are very potent inhibitors of rat liver SAH-hydrolase[12-14] without being substrates of these reactions. Since some of these compounds have an extraordinarily high reversible or irreversible inhibitory effect, we investigated them as possible ligands for affinity chromatography of SAH-hydrolases.

We examined two types of compounds: derivatives containing a carboxy group (eritadenines *Ia–IIIa*) were bonded by an amide bond to 6-aminohexyl-Sepharose, using a soluble carbodiimide, whereas derivatives with a strongly basic amino group on the heterocyclic nucleus or in the side chain (*IVa*, *VI–IXa*) were bonded to 6-carboxyhexyl-Sepharose, using the same condensing agent. After removal of the unreacted derivative, the ligand-to-polymer bonding was easily verified by UV spectra of the polymeric material in aqueous glycerol suspension. These spectra did not differ much from those of the free ligands in the same medium.

The study was carried out with the rat liver enzyme, partly purified by a modified procedure described in one of our previous communications[12]. The polymeric carriers were examined according to the following three aspects: enzyme-to-carrier bond, nonspecific elution of the contaminating proteins, and specific liberation of SAH-hydrolase from the carrier. For analytical experiments the batch method was chosen. The enzymatic activity was estimated by the synthesis of SAH from adenosine and L-homocysteine[12] which was followed using the HPLC technique. The recovered enzyme was not only analysed for its specific activity but also for the presence of contaminating adenosine aminohydrolase and xanthine oxidase activity. In Table I we compare seven new modified polymeric carriers, together with the already described[5] carrier, containing 8-(3-aminopropylamino)adenosine (*IVa*), used as a standard.

The first group of the studied ligands consists of the eritadenines *Ia–IIIa* which belong to the most potent known SAH-hydrolase inhibitors[10,14]. Bonding of both the *erythro*-derivatives *IIa* and *IIIa* (ref.[15]) to 6-aminohexyl-Sepharose 4B afforded the carriers *IIb* and *IIIb* which within several minutes were able to bind all the enzyme from the studied (even dilute) solution. It appeared, however, that the enzyme-carrier bond was so strong that the enzyme could not be liberated by an increase in ionic strength, change of pH, higher temperature or elution with solution of adenosine as substrate/inhibitor. Because of the known fact that in solution the compounds *IIa* and *IIIa* inactivate SAH-hydrolase rapidly and irreversibly[14], a similar inactivation cannot be excluded also in a heterogeneous system (this con-

clusion is contradicted by the observation that substituted amides of compounds *IIa* and *IIIa* lose this activity[16]). It is, however, obvious that carriers of the type *IIb* or *IIIb* are not suitable for affinity purification of SAH-hydrolase; they could possibly be employed for its quantitative removal from biological materials.

Stereoisomeric *threo*-eritadenines (*e.g. Ia*) are substantially weaker SAH-hydrolase inactivators than the *erythro*-isomers *IIa* and *IIIa* (ref.[14]). Sepharose 4B with

TABLE I

Affinity of rat liver SAH-hydrolase toward various modified Sepharose 4B supports (at 4°C, batch procedure)

Support	Elution of enzyme activity			Note[b]
	eluent[a]	*c*, mol l^{-1}	elution	
Ib	A	0·05	–	CP
	A	0·075	–	CP
	A	0·100	+	SE
	A	0·125	+	SE
	A	0·150	+	SE
IIb, *IIIb*	A	0·05–0·20	–	–
	A	0·20–0·50[d]	–	–
	B	4·00[d]	–	–
	C	2·00[d]	–	–
	D	–	–	–
	E	$5 . 10^{-4}$	–	–
IVb, *VIb*	A	0·05–0·15	–	–
	A	0·20	±	DS
	B	1·00–2·00	±	DS
	E	$2·5 . 10^{-4}$	±	DS
VIIb	A	0·05–0·15	–	–
	A	0·20	+	NS
	B	1·00	+	NS
	B	1·50	+	NS
	B	2·00	±	NS
VIIIb, *IXb*	A	0·05–0·20	–	CP
	B	1·00–2·00	–	CP
	E	$2·5 . 10^{-4}$ + B 0·75	±	SS[c]

[a] A Sörensen phosphate buffer pH 7·4; B KCl; C ammonium sulfate; D 4 mol l^{-1} KCl in 0·05 mol. l^{-1} Sörensen phosphate buffer pH 7·4–6·0 (0·5 pH steps); E adenosine; all eluents contain 10^{-3} mol l^{-1} dithiothreitol. [b] CP contaminating proteins; DS diffuse separation; NS non-specific elution; SS sharp separation. [c] SE sharp elution by 2·5–3 column volumes in column runs. [d] At 25°C.

bonded *threo*-isomer *Ia* can be used also for affinity chromatography of the rat liver enzyme. The enzyme is sufficiently strongly bonded to the carrier and the contaminating proteins (including adenosine aminohydrolase and xanthine oxidase) are removed first with gradually increasing ionic strength of the eluate. The purified SAH-hydrolase is liberated only at higher ionic strengths. Affinity column chromatography on this carrier (*Ib*) afforded a highly purified enzyme preparation whose parameters are listed in Table II. The obtained enzyme was homogeneous according to gel electrophoresis, its enzyme activity coinciding with the protein band; its specific activity was very close to the highest values described in the literature[5].

Since the high affinity (and perhaps also inactivation) of the enzyme to the carriers *Ib*–*IIIb* either completely prevents its liberation or, at least, could lower the yields, we turned our attention to reversible SAH-hydrolase inhibitors of the neutral type; such compounds can be adenosine (or its mentioned derivative *IVa*) or 9-(*S*)-(2,3-dihydroxypropyl)adenine (*V*), prepared by us previously[12]. We compared therefore properties of the carriers *IVb* and *VIb* which contain 8-(3-aminopropylamino) derivatives *IVa* and *VIa*. At higher ionic strengths, required for removal of the contaminating proteins, the affinity binding of SAH-hydrolase was not strong enough, causing simultaneous elution of the enzyme. Although SAH-hydrolase was liberated by further elution with adenosine solution, no sharp separation was observed. Both the carriers *IVb* and *VIb* do not differ substantially from each other and, in spite of the reported[5] use of compound *IVa*, this type does not seem to us to be promising.

An alternative model, also simulating a part of the structure *V*, is 9-(*RS*)-(3-(3-aminopropylamino)-2-hydroxypropyl)adenine (*VIIa*). However, the corresponding carrier

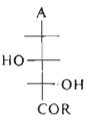
TABLE II

Characteristics of enzyme preparations purified by column affinity chromatography (for conditions see Experimental)

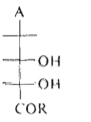
Parameter	Support	
	Ib	*VIIIb*
Specific activity, e.u./mg	4·5	2·0–5·4[a]
K_M^{Ado} (synthesis), mol l^{-1}	$2{\cdot}3 \cdot 10^{-5}$	$7 \cdot 10^{-6}$
V_{max} (synthesis), mol min^{-1}	$1{\cdot}66 \cdot 10^{-6}$ [b]	$0{\cdot}50 \cdot 10^{-6}$ [c]
K_M (hydrolysis), mol l^{-1}	$2 \cdot 10^{-6}$	$2{\cdot}5 \cdot 10^{-6}$
V_{max} (hydrolysis), mol min^{-1}	$0{\cdot}37 \cdot 10^{-6}$ [b]	$0{\cdot}80 \cdot 10^{-7}$ [c]

[a] Depends on the time of storage of the DEAE fraction; the value of 2·0 e.u./mg was measured after storing of the DEAE fraction at −70°C for 17 months; [b] 0·36 μg enzyme protein per ml; [c] 0·16 μg enzyme protein per ml.

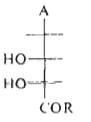
VIIb presents also no advantage over those with 8-substituted adenine nucleus (*IVb*, *VIb*). With increasing ionic strength, SAH-hydrolase is gradually liberated, without any specific separation.



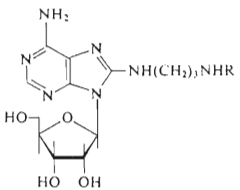
Ia, R = OH
Ib, R = NH(CH$_2$)$_6$Ⓟ



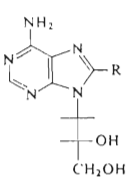
IIa, R = OH
IIb, R = NH(CH$_2$)$_6$Ⓟ



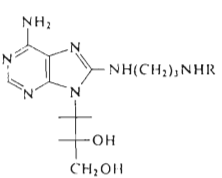
IIIa, R = OH
IIIb, R = NH(CH$_2$)$_6$Ⓟ



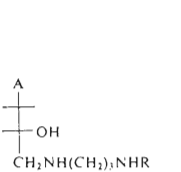
IVa, R = H
IVb, R = CO(CH$_2$)$_6$Ⓟ



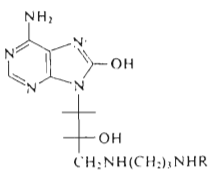
V, R = H
X, R = OH



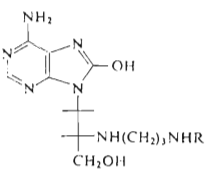
VIa, R = H
VIb, R = CO(CH$_2$)$_6$Ⓟ



VIIa, R = H
VIIb, R = CO(CH$_2$)$_6$Ⓟ



VIIIa, R = H
VIIIb, R = CO(CH$_2$)$_6$Ⓟ



IXa, R = H
IXb, R = CO(CH$_2$)$_6$Ⓟ

In formulae, A represents adenin-9-yl, Ⓟ Sepharose 4 B polymeric support.

The most suitable compounds which we encountered so far were two derivatives related to the compound *VIIa*: the isomeric 9-substituted 8-hydroxyadenine derivatives with aliphatic propyl chain, carrying one hydroxy and one 3-aminopropylamino

group in positions 2 and 3. These compounds *VIIIa* and *IXa* were covalently bonded to the polymeric matrix by the amino group in the side chain on treatment with a soluble carbodiimide (under the conditions used, no side-reaction of the heterocyclic system with the carbodiimide takes place). The affinity carriers *VIIIb* and *IXb* satisfy fully the expected requirements: the enzyme-carrier bonding is strong enough to allow removal of the contaminating proteins by elution with concentrated electrolyte solutions and the pure SAH-hydrolase is liberated specifically with adenosine solution. The applicability of both these affinity carriers has been repeatedly proved on a preparative scale (Figs 1 and 2). After removal of adenosine by gel filtration, SAH-hydrolase of high specific activity and free of contaminating proteins is obtained in good yield. Its kinetic parameters are close to those of the enzyme obtained using the carrier *Ib* (Table II).

The described high efficiency of both carriers with ligands of 8-hydroxyadenine structure is unexpected: neither 9-(*RS*)-(2,3-dihydroxypropyl)-8-hydroxyadenine (*X*) nor the ligands *VIIIa* and *IXa* alone inhibit measurably SAH-hydrolase *in vitro* (Table III). The absence of affinity toward an enzyme on binding the inhibitor

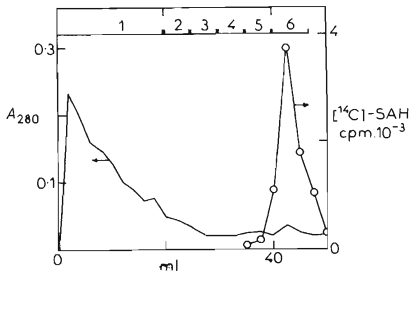


FIG. 1

Affinity chromatography of S-adenosyl-L-homocysteine hydrolase. The column of *VIIIb* (3 × 0·8 cm) was loaded with 0·700 mg (DEAE-fraction) and elution performed as described in Experimental (*cf.* Table I).
1 A 0·01, *2* A 0·20, *3* B 1·00, *4* B 1·50, *5* B 2·00, *6* E 2·5 . 10^{-4} + B 0·75. Concentration in mol . l^{-1}.

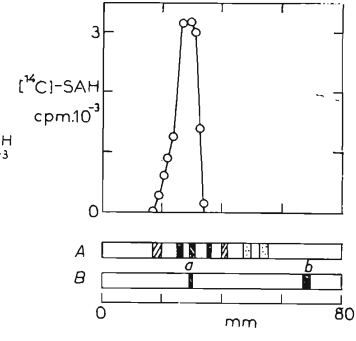


FIG. 2

Polyacrylamide gel electrophoresis of S-adenosyl-L-homocysteine hydrolase. *A* DEAE-fraction, *B* enzyme purified on *VIIIb* column (*a*), supplemented with bovine serum albumine (*b*). [^{14}C]-SAH cpm. 10^{-3} enzym. activity

to the polymer support is often encountered; however, the appearance of affinity on binding a non-inhibitor is rather surprising.

The described method of SAH-hydrolase purification on carriers of the type *VIIIb* and *IXb* proved to be advantageous not only in isolation of the enzyme from rat liver but also in a very efficient purification of SAH-hydrolases from other plant or animal tissues on both large and very small scale (*e.g.* from several milligrams of the tissue[10]). Both the ligands *VIIIa* and *IXa* (or their mixture) are relatively easily accessible[17], the carriers prepared from them are sufficiently stable under normal conditions (*i.e.* storing in salt solutions at 0°C) and their separating capacity is relatively high. In most cases, particularly in small-scale experiments, a very efficient separation of SAH hydrolases from other proteins can be achieved on these carriers without previous fractionation of the mixture obtained by extraction of the biological material[18].

EXPERIMENTAL

Chemicals. The ligands *Ia*—*IIIa* were synthesized according to ref.[15] and were used as sodium salts. Compounds *IIa*, *VIa*—*IXa* and *X* were prepared by the described procedure[17]; all the ligands were homogeneous according to the criteria given in the original papers. N-Cyclohexyl--N'-(3-trimethylammoniumpropyl)carbodiimide *p*-toluenesulfonate was prepared as described in ref.[19].

Methods. UV spectra were measured in aqueous solutions or in 30% aqueous glycerol on a Specord UV-VIS (Zeiss, Jena, G.D.R.) instrument. Protein determination was carried out with Coomassie Brilliant Blue at 607 nm as described in ref.[20]. Disc-gel electrophoresis in polyacrylamide gel was performed according to ref.[21] in 5% (w/v) separation gel (length 80 mm) in 0·375

TABLE III

Inhibition of the rat-liver SAH-hydrolase by compounds *Ia*—*X*

Compound	v_i/v_0	Compound	v_i/v_0
Ia	0·50	*VIa*	0·97
IIa	0·30[a]	*VIIa*	1·00
IIIa	0·36[b]	*VIIIa*	1·00
IVa	0·85	*IXa*	1·00
V	0·63	*X*	1·00
IIa-ethylamide	0·90		

In the hydrolytic reaction; $[SAH] = 4 . 10^{-6}$ mol l^{-1}, $[I] = 10^{-6}$ mol l^{-1}. $K_M = 2·5 . 10^{-6}$ mol l^{-1}; for conditions *cf.*[12].

[a] $[I] = 10^{-8}$ mol l^{-1}; [b] $[I] = 10^{-7}$ mol l^{-1}.

mol l^{-1} TRIS-HCl buffer pH 8·9 using a 2·5% stacking gel (25 mm length) in 0·125 mol l^{-1} TRIS-HCl buffer pH 6·7. Electrophoresis was carried out in 40 mmol l^{-1} TRIS-glycine buffer pH 8·3 at 180—200 V (2 mA per tube) for 3 h at 4°C. SAH-hydrolase in the polyacrylamide gel was detected in 3 mm-thick slices of gel after incubation in the standard reaction mixture (*vide infra*); the protein position was located by comparison with a gel stained with Coomassie Brilliant Blue R 250. The SAH hydrolase activity was determined either with use of radioactive substrates (SAH or adenosine) according to the previously described method[12] (Method *A*) or by HPLC analysis of the total reaction mixture of the same composition[12] containing non-labeled substrates. The HPLC analysis was carried out on a 3·3 × 150 mm column of Separon SI C18 (5 μ); elution (0·4 ml/min) with 0·01 mol l^{-1} ammonium dihydrogen phosphate pH 2·8, containing 10%(wt/wt) of methanol; detection at 254 nm, sensitivity 0·02—0·08 absorbancy units per full scale.

Enzyme preparation. Livers (20 g) of 3 months old female Wistar A rats were used for the SAH-hydrolase isolation. The enzyme was purified according to the method of de la Haba and Cantoni[22] up to the point of ammonium sulfate precipitation (0—80% saturation). The protein precipitate was then gel-filtered through a Sephadex G 25 coarse column (3 × 50 cm) in 0·01 mol . l^{-1} potassium phosphate buffer pH 7·37. The enzyme fraction was further purified by column chromatography on DEAE cellulose (2·2 × 5·5 cm) with potassium chloride concentration gradient (0—0·4 mol l^{-1}) in the same buffer. SAH-Hydrolase of specific activity 0·9 e.u. per mg protein was eluted at 0·1 mol l^{-1} KCl concentration. This enzyme preparation (total yield 2·6 mg protein, 65 μg protein/ml) was diluted (1 : 3) with 0·01 mol l^{-1} potassium phosphate buffer pH 7·37 containing 0·001 mol l^{-1} dithiothreitol and used further for affinity chromatography experiments ("DEAE-fraction").

Bond of the Ligands to CH- or AH-Sepharose 4B

Freeze-dried AH or CH-Sepharose 4B (1 g; 4 ml of swollen gel) was shaken overnight with 0·5 mol l^{-1} sodium chloride solution (50 ml). The suspension was filtered and washed with 200 ml of the same solution, followed with water (200 ml). The wet gel was resuspended in water (8 ml), briefly evacuated and the ligand (112 μmol *IVa*, *VIa*—*IXa* to CH-Sepharose or 80 μmol *Ia*—*IIIa* to AH-Sepharose) was added. The mixture was then adjusted to pH 5·0 with dilute hydrochloric acid and solid N-cyclohexyl-N'-(3-trimethylammoniumpropyl)carbodiimide *p*-toluenesulfonate (1·12 mmol) was added under stirring. The mixture was stirred at room temperature, pH being kept at 5·0—5·5 by addition of 2 mol l^{-1} hydrochloric acid during the first 30 min of the reaction. Another 1·1 mmol portion of the condensing agent was added, the mixture adjusted to pH 5·0 (see above) and gently shaken overnight at room temperature. The gel was filtered, washed with 0·5 mol l^{-1} sodium chloride solution (total 500 ml) and water (500 ml) and stored in saturated sodium chloride solution at +4°C.

Affinity Chromatography

Batch method. The polymeric support (0·2 ml, prewashed with 0·01 mol l^{-1} Sörensen phosphate buffer pH 7·37) was added to the DEAE-fraction (0·5 ml) in an Eppendorff polypropylene microtube and kept for 20 min in an ice-bath. The suspension was centrifuged and an aliquot of the supernatant was used for the SAH-hydrolase estimation (Method *B*). The supernatant was then removed by a Pasteur pipette and replaced by the appropriate elution solution. The protein concentration in the supernatants was determined by UV measurement at 280 nm.

Column method. The DEAE fraction (20—40 ml) was applied in five 5 ml-portions on a column (3 × 0·8 cm) of the modified Sepharose equilibrated with 0·01 mol l^{-1} Sörensen phosphate

buffer pH 7·37, containing dithiothreitol (0·001 mol l^{-1}). The column was then washed discontinually with 3—5 ml portions of precooled eluents (*cf.* Table I). The whole operation was performed at 4°C. The activity of SAH-hydrolase in the corresponding fractions (2·5 ml each) was estimated by methods *A* and *B*, the protein content by UV spectroscopic measurement at 280 nm.

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