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SHORT COMMUNICATION

Effects of Ions on Adenosine Binding and Enzyme Activity of Purified *S*-Adenosylhomocysteine hydrolase from Bovine Kidney

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ABSTRACT. The present investigation was undertaken to determine the effect of various ions on the characteristics of *S*-adenosylhomocysteine (SAH) hydrolase from bovine kidney. The binding sites of [^{3}H]-adenosine to purified SAH hydrolase were not influenced by phosphate, magnesium, potassium, sodium, chloride or calcium ions at physiological cytosolic concentrations. To test whether NAD^+ in the SAH hydrolase is essential for adenosine binding, we prepared the apoenzyme by removing NAD^+ with ammonium sulfate. The resulting apoenzyme did not exhibit any [^{3}H]-adenosine binding. Since the apoenzyme was enzymatically inactive, it is suggested that adenosine binds to the active site and not to an allosteric site of the intact enzyme. The kinetics of the hydrolysis and the synthesis of SAH catalyzed by the enzyme SAH hydrolase were measured in the presence and absence of phosphate and magnesium. Phosphate increased the V_{max} for both synthesis and hydrolysis. However, only the affinity of adenosine for SAH synthesis was significantly enhanced from 10.1 ± 1.3 μM to 5.4 ± 0.5 μM by phosphate. This effect was already maximal at a phosphate concentration of 1 mM. All other tested ions were without effect on the enzyme activity. Our results show that phosphate at physiological concentrations shifts the thermodynamic equilibrium of SAH hydrolase in the direction of SAH synthesis. These findings imply that SAH-sensitive transmethylation reactions are inhibited during renal hypoxia when intracellular levels of phosphate, adenosine, and SAH are elevated. BIOCHEM PHARMACOL **56**;11: 1493–1496, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. *S*-adenosylhomocysteine hydrolase; apoenzyme; intracellular adenosine binding; phosphate; thermodynamic equilibrium

S-Adenosyl-L-homocysteine hydrolase (EC 3.3.1.1) catalyzes the reversible hydrolysis of SAH† to adenosine and homocysteine [1]. It plays a central role in the regulation of the tissue level of SAH [2–4] and may indirectly influence biological methylation reactions such as capping of mRNA [5] via intracellular SAH accumulation. Since inhibitors of SAH hydrolase have been shown to have antiviral properties [6, 7], it appears desirable to study basic adenosine SAH hydrolase interactions in more detail in order to develop antiviral drugs that inhibit SAH hydrolase. In addition, topically applied adenosine has been found to exhibit antiherperic actions [8].

The equilibrium of the reaction favors the synthesis of SAH with a K_{eq} of 10^{-6} M [1]. However, hydrolysis of SAH to adenosine and L-homocysteine prevails under physiological conditions because both reaction products are removed rapidly, adenosine by adenosine kinase or adenosine deaminase and L-homocysteine by methionine synthase or cystathionine β-synthase [1]. As an adenosine binding protein [9], SAH hydrolase also regulates intracellular free adenosine concentration. SAH hydrolase contains tightly bound NAD^+, 4 moles of NAD^+/mol of enzyme, in agreement with the number of active sites. Palmer and Abeles [10] have proposed a mechanism of action of the hydrolase in which oxidation-reduction of the enzyme-bound NAD^+ plays an integral part in the catalytic pathway.

As recently shown [11], purified SAH hydrolase from bovine kidney exhibits three [^{3}H]-adenosine binding sites which have different affinities. Binding experiments with ^{3}H-adenosine demonstrate that approximately two moles of adenosine bind to one mole of SAH hydrolase. From this binding, a fraction of 20–25% of adenosine is tightly bound to SAH hydrolase. In the present study, we examined the interference of various ions on the adenosine binding and enzyme activity of purified SAH hydrolase from bovine kidney. To gain further insight into the mode of binding of adenosine to the enzyme-protein, we prepared the apoenzyme and examined its interaction with NAD^+.

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† *Abbreviation:* SAH, *S*-adenosylhomocysteine.

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MATERIALS AND METHODS

Materials

The following materials were purchased from the sources indicated: [2,8,5′-^{3}H]-adenosine (2.3 TBq/mmol) NEN; adenosine and *S*-adenosylhomocysteine, Boehringer Mannheim; choline chloride, Boehringer Ingelheim; sodium tetrathionate dihydrate and KNO_3, Fluka. All other chemicals were obtained from Merck.

Enzyme Purification

SAH hydrolase was purified from bovine kidney using chromatographical techniques as described previously [11]. The purified enzyme was frozen at −20° until use.

Binding Assay Procedure

Binding experiments were performed in a final volume of 300 μL 20 mM Tris/40 mM Hepes buffer, pH 7.4 with a concentration of SAH hydrolase of 10 μg/mL, fixed concentrations of [^{3}H]-adenosine (25 nM) and various concentrations (0–175 mM) of phosphate, magnesium, potassium, sodium, chloride, and calcium in Tris/Hepes buffer, pH 7.4. After incubation (4 hr at 20°), samples were filtered, and the radioactivity adsorbed on the filters was determined by liquid scintillation counting.

Preparation of Apo-SAH Hydrolase

The apoenzyme of SAH hydrolase was prepared at saturated ammonium sulfate concentrations as described by Gomi *et al.* [12]. The protein was dissolved in 20 mM Tris/40 mM Hepes buffer, pH 7.4 and desalted over dialysis.

Enzymatic Activity of SAH Hydrolase

The enzyme activity was assayed in both the hydrolytic and synthetic reactions of SAH.

Spectrophotometric Titration of the Enzyme

Reduction of the SAH hydrolase-bound NAD^+ by adenosine causes an increase in the absorbance at 327 nm [11, 13]. The enzyme (0.45 mg/mL) was titrated with adenosine in 20 mM of potassium phosphate, pH 7.4 and in 20 mM of Tris/Hepes, pH 7.4 at room temperature. The titration data were fitted using the following equation with the assumption that the stoichiometry for adenosine binding was 1/subunit,

$$A = \epsilon \cdot ES \tag{1}$$

where A is the absorbance induced by the enzyme–adenosine complex, ϵ is the molar absorbance coefficient, and ES is the SAH hydrolase–adenosine complex. Substituting ES by free and bound adenosine and free enzyme and enzyme–adenosine complex, respectively, yielded

$$A = \epsilon\left[\frac{S_t + E_t + K_{ap}}{2} - \sqrt{\left(\frac{S_t + E_t + K_{ap}}{2}\right)^2 - E_t S_t}\right] \tag{2}$$

which was used to fit the titration data. S_t is the concentration of bound and free adenosine, E_t is the concentration of enzyme–adenosine complex and free enzyme, and K_{ap} is the dissociation constant of the enzyme–adenosine complex.

Data Calculation and Statistics

Student's *t*-test for unpaired values was used to determine the levels of significance. The data were analyzed using nonlinear regression analysis. The run test was used to determine the correctness of the data fit to a given curve. The F test was applied to compare curve fitting for a one versus two or more receptor site model, and P values of <0.05 were considered significant.

RESULTS

Binding Characteristics

To determine the influence of ions on [^{3}H]-adenosine binding to purified SAH hydrolase from bovine kidney, competition studies were performed. Phosphate, magnesium, chloride, and potassium ions were without effect on [^{3}H]-adenosine binding, whereas $CaCl_2$ was found to affect [^{3}H]-adenosine binding with an EC_{50} of 75 mM. Sodium reduced the specific [^{3}H]-adenosine binding by 50% with an EC_{50} of 45 mM.

Kinetic Parameters of the Enzyme Catalysis

The enzyme activity exhibited in the hydrolytic direction, in Tris/Hepes buffer, a Michaelis–Menten kinetic with a K_m of 12.5 μM for SAH and a V_{max} of 0.28 μmol/min/mg. In the direction of synthesis, the SAH hydrolase activity was characterized by a K_m of 10.1 μM for adenosine and a V_{max} of 0.57 μmol/min/mg under the same buffer conditions. P_i stimulated the initial velocity of the reaction of both the hydrolytic and synthetic directions. The catalytic activity of the enzyme increased in the presence of phosphate in the hydrolytic and synthetic direction by about 30–36% (Table 1). In the direction of synthesis, the catalytic activity of the enzyme was already maximal at a phosphate concentration of 1 mM, whereas in the hydrolytic direction 1 mM of phosphate did not increase the enzyme activity. However, in the presence of phosphate, the K_m for synthesis was reduced by about the factor 2 from 10.1 to 5.4 μM, while the K_m for the hydrolysis was not affected.

The other divalent cations, magnesium and calcium as well as the monovalent anion chloride and the cations sodium and potassium showed no significant effect on the

TABLE 1. Effect of phosphate on K_m and V_{max} of the purified SAH hydrolase

	Tris/Hepes pH 7.5		Phosphate pH 7.4	
	K_m (μM)	V_{max} (μmol min^{-1} mg^{-1})	K_m (μM)	V_{max} (μmol min^{-1} mg^{-1})
Synthesis	10.1 ± 1.3	0.57 ± 0.02	5.4* ± 0.4	0.82* ± 0.08
Hydrolysis	12.5 ± 1.1	0.28 ± 0.03	10.3 ± 1.0	0.37 ± 0.04

In the direction of SAH *hydrolysis*, the decrease in SAH concentration (1–100 μM) was measured photometrically at 265 nm with SAH hydrolase (20 μg/mL) and adenosine deaminase (2U/mL). In the direction of SAH *synthesis*, adenosine (0.5–200 μM), L-homocysteine (500 μM) and SAH hydrolase (20 μg/mL) were incubated at 20°. After stopping the reaction with 50 μL 0.6 N perchloric acid and centrifugation, SAH concentration was determined in 50 μL of supernatant by HPLC. Values are means of four experiments ± SEM.

* $P < 0.05$ for comparison between Tris/Hepes and phosphate buffer.

catalytic activity, either in the synthetic or in the hydrolytic direction.

Kinetics of the Apo-SAH hydrolase

The acid ammonium sulfate precipitation removed the NAD^+ from the covalent binding of SAH hydrolase and led to the apoenzyme and NAD^+. A gel filtration was performed to demonstrate that the apoenzyme retained its tetrameric structure. The apoenzyme showed no [^{3}H]-adenosine binding and no enzyme activity.

Titration of SAH hydrolase by Adenosine

Adenosine reduced enzyme-bound NAD^+ in a "half-reaction" to form reduced enzyme-bound NADH that does not complete a catalytic cycle in the absence of L-homocysteine. The reduced NADH exhibits an absorption maximum at 327 nm [13]. The spectrophotometric titration of the enzyme with adenosine is shown in Fig. 1. From these data, one can calculate the equilibrium constant of adenosine with SAH hydrolase and its molar ratio. The titration data fitted to equation 2 gave an equilibrium constant of adenosine of 240 ± 20 nM in phosphate buffer and 960 ± 40 nM in Tris/Hepes, respectively. The molar absorbance coefficient for the enzyme-bound NADH calculated from both titration curves was 2.512 M^{-1} cm^{-1}.

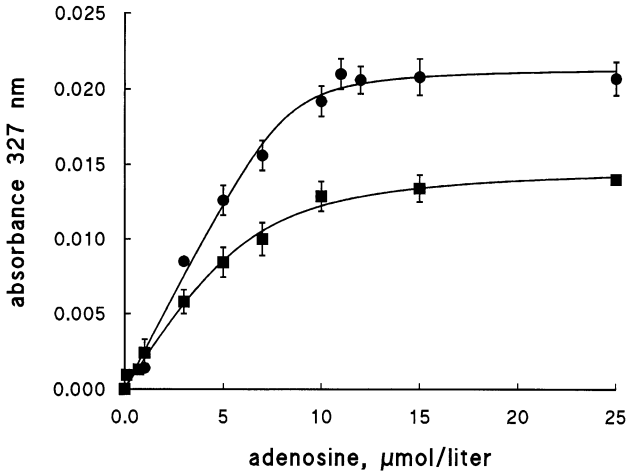

FIG. 1. Titration of SAH hydrolase with adenosine. The titration of 2.36 μM enzyme with adenosine was monitored spectrophotometrically with an emission wavelength of 327 nm in 20 mM of potassium phosphate (●) (pH 7.4) and in 20 mM of Tris/Hepes (■) (pH 7.4) at room temperature, respectively. Values represent means ± SEM (N = 3).

The intersection of the initial slope at low concentrations with the asymptote at saturating concentrations of adenosine corresponded to 3.8 mol of adenosine added per 1 mol SAH hydrolase in 20 mM of potassium phosphate. Under these buffer conditions, 4 NAD^+ per mol SAH hydrolase was transformed into the reduced form. In the absence of phosphate, only 78% of the enzyme-bound NAD^+ was transformed into the reduced form.

DISCUSSION

SAH hydrolase from bovine kidney binds [^{3}H]-adenosine with a high affinity. In the present study, we wanted to further analyze the nature of this binding. Of the ions investigated, only sodium and calcium showed an effect on [^{3}H]-adenosine binding. Since SAH hydrolase is an intracellular enzyme and since the inhibiting concentrations of these ions are above physiological cytosolic concentrations, it is unlikely that the effect of both ions presents a physiologically important finding. However, during hypoxia of the cells, sodium can increase up to inhibitory concentrations (50 mM).

Phosphate stimulated the enzyme activity in both directions by about 30%. Whereas in the direction of hydrolysis the Michaelis–Menten constant was not affected, in the direction of hydrolysis the K_m was reduced by the factor 2 (Table 1), indicating that the synthesis is favored in phosphate buffer. With the spectrophotometric assay (reduction of NAD^+ to NADH), we found, in the presence of phosphate, a molar ratio of adenosine/SAH hydrolase of 4:1 (Fig. 1), which is in good agreement with the findings reported by Palmer and Abeles [10] and Fujioka and Takata [14]. The fact that the apoenzyme did not bind [^{3}H]-adenosine shows that NAD^+/NADH is directly involved in the binding mechanism. This finding together with the fact of single-site binding isotherms [11] strongly favors the conclusion that adenosine binds at the active site of the enzyme and not at an allosteric site. In the absence of phosphate (Tris/Hepes buffer), the molar ratio of adeno-

sine/SAH hydrolase was found to be 3:1. This might indicate that phosphate can facilitate the efficiency of conversion of NAD^+ to NADH by adenosine.

From the phosphate effect on the SAH synthesis rate, it can be predicted that increasing free phosphate concentrations in the cytosol above physiological levels of 0.6 mM [15] would lead to elevated cytosolic SAH concentrations. Simultaneous increases in intracellular adenosine concentrations as seen in hypoxic tissue would strongly favor an enhanced SAH synthesis rate. Indeed, we found a 10-to-20-fold increase in SAH tissue content parallel to adenosine accumulation after short periods of renal ischemia (0.5–10 min) when the ATP hydrolysis rate prevails over the ATP synthesis rate [16, 17]. It can be assumed that during renal hypoxia SAH-sensitive transmethylation reactions are inhibited. It is interesting to note that SAH hydrolase inhibitors which increase the intracellular SAH concentration have been shown to possess strong antiviral activity [18, 19].

In summary, our data show: (1) adenosine binds to the active site of the enzyme; (2) phosphate facilitates adenosine-mediated conversion of NAD^+ to NADH; and (3) phosphate at cytosolic concentrations shifts the thermodynamical equilibrium further in the direction of synthesis.

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References

1. de la Haba G and Cantoni GL, The enzymatic synthesis of *S*-adenosyl-L-homocysteine from adenosine and homocysteine. *J Biol Chem* **234:** 606–608, 1959.
2. Kredich NM and Hershfield MS, *S*-adenosylhomocysteine toxicity in normal and adenosine kinase-deficient lymphoblasts of human origin. *Proc Natl Acad Sci USA* **76:** 2450–2454, 1979.
3. Ueland PM, Pharmacological and biochemical aspects of *S*-adenosylhomocysteine and *S*-adenosylhomocysteine hydrolase. *Pharmacol Rev* **34:** 223–253, 1972.
4. Ueland PM and Helland S, Binding of adenosine to intracellular *S*-adenosylhomocysteine hydrolase in isolated rat hepatocytes. *J Biol Chem* **258:** 747–752, 1983.
5. Shatkin AJ, Capping of eucaryotic mRNAs. *Cell* **9:** 645–653, 1976.
6. Cools M, Balzarini J and De Clercq E, Mechanism of antiviral and cytotoxic action of (±)-6′ β-fluoroaristeromycin, a potent inhibitor of *S*-adenosylhomocysteine hydrolase. *Mol Pharmacol* **39:** 718–724, 1991.
7. Liu S, Wnuk SF, Yuan C, Robins MJ and Borchardt RT, Adenosine-5′-carboxaldehyde: A potent inhibitor of *S*-adenosyl-L-homocysteine hydrolase. *J Med Chem* **36:** 883–887, 1993.
8. Osswald H, Topical composition for treating herpes with adenosine. *USP* 5034382, 1991.
9. Hershfield MS and Kredich NM, *S*-adenosylhomocysteine hydrolase is an adenosine binding protein: A target for adenosine toxicity. *Science* **202:** 757–760, 1978.
10. Palmer JL and Abeles RH, The mechanism of action of *S*-adenosylhomocysteinase. *J Biol Chem* **254:** 1217–1225, 1978.
11. Kloor D, Fuchs S, Kurz J, Faust B and Osswald H, *S*-adenosylhomocysteine-hydrolase from bovine kidney: Enzymatic and binding properties. *Kidney and Blood Pressure Res* **19:** 100–108, 1996.
12. Gomi T, Takata Y and Fujioka M, Rat liver *S*-adenosylhomocysteinase. Spectrophotometric study of coenzyme binding. *Biochim Biophys Acta* **994:** 172–179, 1989.
13. Matuszewska B and Borchardt RT, The role of nicotineamide adenine dinucleotide in the inhibition of bovine liver *S*-adenosylhomocysteine hydrolase by neplanocin A. *J Biol Chem* **262:** 265–268, 1987.
14. Fujioka M and Takata Y, *S*-adenosylhomocysteine hydrolase from rat liver. Purification and some properties. *J Biol Chem* **256:** 1631–1635, 1981.
15. Freeman D, Bartlett S, Radda G and Russ B, Energetics of sodium transport in the kidney. Saturation transfer 31P-NMR. *Biochem Biophys Acta* **762:** 325–336, 1983.
16. Kloor D, Stumvoll W, Faust B, Delabar D, Mühlbauer B and Osswald H, Does *S*-adenosyl-homocysteine (SAH) accumulate in the ischemic rat kidney? *Naunyn-Schmiedeberg's Arch Pharmacol* **353:** R11, 1996.
17. Osswald H, Schmitz HJ and Kemper R, Tissue content of adenosine, inosine and hypoxanthine in the rat kidney after ischemia and postischemic recirculation. *Pflügers Arch* **371:** 45–49, 1977.
18. De Clercq E, *S*-Adenosylhomocysteine hydrolase inhibitors as broad-spectrum antiviral agents. *Biochem Pharmacol* **36:** 2567–2575, 1987.
19. Daelemans D, Este JA, Witvrouw M, Pannecouque C, Jonckheere H, Aquaro S, Perno CF, De Clercq E and Vandamme AM, *S*-adenosylhomocysteine hydrolase inhibitors interfere with the replication of human immunodeficiency virus type 1 through inhibition of the LTR transactivation. *Mol Pharmacol* **52:** 1157–1163, 1997.