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Characterization of the cAMP binding site of purified *S*-adenosyl-homocysteine hydrolase from bovine kidney

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Abstract

The enzyme *S*-adenosyl-homocysteine hydrolase (AdoHcyase) which catalyzes the reversible hydrolysis of AdoHcy to adenosine and homocysteine is an adenosine binding protein. In the present study we examined the characteristics of [^{3}H]cAMP binding to purified AdoHcyase from bovine kidney in comparison with the high affinity adenosine binding site of AdoHcyase. AdoHcyase exhibits one [^{3}H]cAMP binding site with an affinity of $K_d = 23.1 \pm 1.1$ nM and a B_{max} of 116.6 ± 3.8 pmol/mg protein. Binding of [^{3}H]cAMP obeyed a monophasic reaction with a k_{+1} value of 0.035 min/M. The dissociation of AdoHcyase–[^{3}H]cAMP complex exhibited a time- and temperature-dependent character. After a 240 min incubation at 0° only 5–10%, however, at 20° 90% were displaceable. Adenosine and cAMP displace each other with similar affinities of EC_{50} 57 nM vs. EC_{50} 65 nM. 2′-Deoxyadenosine, N^6-methyladenosine, and NECA displace 25 nM [^{3}H]cAMP and 10 nM [^{3}H]adenosine with EC_{50} values of 94, 90 and 80 nM, respectively. All other nucleosides studied, adenine, inosine, adenosine-2′,3′-dialdehyde, 2-chloroadenosine, aristeromycin, and adenine nucleotides were only week competitors for [^{3}H]cAMP and [^{3}H]adenosine. These compounds displace [^{3}H]cAMP and [^{3}H]adenosine with equal potencies. Our data indicate that the binding site for nanomolar concentrations of cAMP and adenosine at the AdoHcyase appears to be identical. The physiological implications of a cAMP binding site at the AdoHcyase remain to be established.

Keywords: *S*-Adenosyl-homocysteine hydrolase; Adenosine; cAMP binding protein; cAMP binding site; Adenosine analogues; NAD^+/NADH ratio

1. Introduction

AdoHcyase (EC 3.3.3.1) catalyzes the reversible hydrolysis of AdoHcy to adenosine and homocysteine and thus regulates *S*-adenosylmethionine-dependent transmethylation reactions by hydrolyzing the potent feedback inhibitor AdoHcy [1]. The equilibrium of the reaction favors the synthesis of AdoHcy with a K_{eq} of 10^{-6} M [2]. However, hydrolysis of AdoHcy to adenosine and homocysteine prevails under physiological conditions because both reaction products are removed rapidly, adenosine by adenosine kinase or adenosine deaminase and homocysteine by cystathionine β-synthase or 5-methyltetrahydrofolalate-homocysteine *S*-methyltransferase.

As recently shown AdoHcyase binds adenosine with three different affinities and a capacity of 2 mol adenosine/mol enzyme. Therefore, AdoHcyase is one of the adenosine binding proteins in the kidney [3]. AdoHcyase contains tightly bound NAD^+, 4 mol of NAD^+/mol enzyme which plays an integral part in the catalytic pathway [4,5]. The extent of NAD^+ reduction in the enzyme determines the adenosine binding and enzyme activity of AdoHcyase [6].

The concept of cAMP as a second messenger implies that cAMP mediates not only a wide variety of cellular responses to hormonal signals, including changes in intermediary metabolism but also modulates processes of cellular proliferation and cellular motility [7–10]. Specific intracellular cAMP binding proteins, which are not associated with cAMP-dependent protein kinase, may have a possible function to modulate the effect of intracellular cAMP [11]. Earlier reports have shown that AdoHcyase is also a cAMP binding protein [11–14]. The binding of cAMP to AdoHcyase was reported to be weaker compared to that of adenosine and can be inhibited by the latter [14]. Since cAMP increases the activity of AdoHcyase in the

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Abbreviations: AdoHcy, *S*-adenosyl-homocysteine; AdoHcyase, *S*-adenosyl-homocysteine hydrolase; AdoMet, *S*-adenosylmethionine; NECA, 5′-*N*-ethylcarboxamidoadenosine; DPCPX, 8-cyclopentyl-1,3-dipropyl-xanthine.

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direction of hydrolysis [15] and since AdoHcy was suggested to inhibit cAMP phosphodiesterase [16], the physiological effect of cAMP may serve to maintain AdoHcy tissue content at a low level.

Although AdoHcyase binds adenosine and cAMP little information is available whether there is a common binding site on AdoHcyase which is shared by adenosine and cAMP. In the present study we characterize the cAMP binding site and the interference of various adenosine analogues on adenosine and cAMP binding on purified AdoHcyase from bovine kidney.

2. Materials

The following materials were purchased from the sources indicated: [2,8-^{3}H]adenosine-3′,5′-cyclic phosphate (925 GBq/mmol), [2,8,5′-^{3}H]adenosine (2.3 TBq/mmol) NEN Germany; adenosine, cAMP, AMP, ATP, Boehringer; adenine, 2′-deoxyadenosine, adenosine-2′,3′-dialdehyde, aristeromycine, 2-chloroadenosine, N^6-methyladenosine, diadenosine-diphosphate, 3-deazaadenosine, inosine, hypoxanthine, Sigma–Aldrich; NECA, theophylline, DPCPX RBI. All buffer chemicals were of the highest available quality exclusively from Merck.

3. Methods

3.1. Enzyme purification

AdoHcyase was purified from bovine kidney with chromatographical techniques as described previously [3]. The purified enzyme was frozen at −20° until use.

3.2. Protein assay

Protein concentration was determined according to the method of Bradford [17] using bovine serum albumin as a standard.

3.3. 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 AdoHcyase of 10 μg/mL. Association kinetic studies were performed by incubating AdoHcyase with 25 nM [^{3}H]cAMP at 0 and 20°. Samples of the reaction mixture were removed after 5, 10, 15, 30, 60, 90, 120, 180, 240, 300 min after addition of [^{3}H]cAMP and filtered through GF/B filters (Whatman) presoaked for at least 1 hr in 0.3% polyethylenimine [18]. The filters were washed with 8 mL of ice cold 50 mM Tris–HCl buffer pH 7.4 and 1 mM DTT. The filtration and rinsing process was finished in 5 s. Radioactivity adsorbed on the filters was determined by liquid scintillation counting.

Dissociation kinetic studies were performed after equilibrium binding of [^{3}H]cAMP (25 nM) to AdoHcyase by incubation for 240 min at 0 and 20°. The dissociation reaction was initiated by addition of unlabeled cAMP in a final concentration of 1 mM. Samples of the reaction mixtures were removed before and at various times after addition of cAMP and were filtered through GF/B filters, as described above.

For saturation analysis, [^{3}H]cAMP was included at final ligand concentrations of 1.0–300 nM. The nonspecific binding was determined in the presence of 1 mM unlabeled cAMP. The reaction mixture was incubated for 2 hr at 20°. The AdoHcyase–[^{3}H]cAMP complex was recovered by rapid filtration through Whatman GF/B filters as described above.

In competition experiments, fixed concentrations of [^{3}H]cAMP (25 nM) and [^{3}H]adenosine (10 nM) were incubated for 2 hr at 20° with concentrations (0–10^{-4} M) of adenosine analogues in 20 mM Tris–40 mM Hepes buffer pH 7.4. After incubation samples were filtered and washed as described above.

3.4. Preparation of apo-AdoHcyase

The apoenzyme of AdoHcyase was prepared at saturated ammonium sulfate concentration as described by Gomi *et al.* [19]. The protein was dissolved in 20 mM Tris–40 mM Hepes buffer pH 7.4 and desalted over dialysis.

3.5. Spectrophotometric titration of the enzyme

Reduction of the AdoHcyase bound NAD^+ by adenosine causes an increase in the absorbance at 327 nm [5,20]. The enzyme (0.47 mg/mL) was titrated with adenosine and cAMP in 20 mM potassium phosphate pH 7.4 at room temperature. The titration data were fitted as described previously [5].

4. Calculation and statistics

Data were analyzed using two fitting procedures, LIGAND [21] and nonlinear regression analysis. The run test was used to determine the goodness of fit data to a given curve. The *F* test was applied to compare curve fitting for a one vs. two or more receptor site model, *P* values of <0.05 were considered significant.

5. Results

5.1. Binding of cAMP

At 0° the association of 25 nM [^{3}H]cAMP was not completed after 300 min and only 5–10% dissociated within 240 min after addition of 1 mM nonlabeled cAMP.

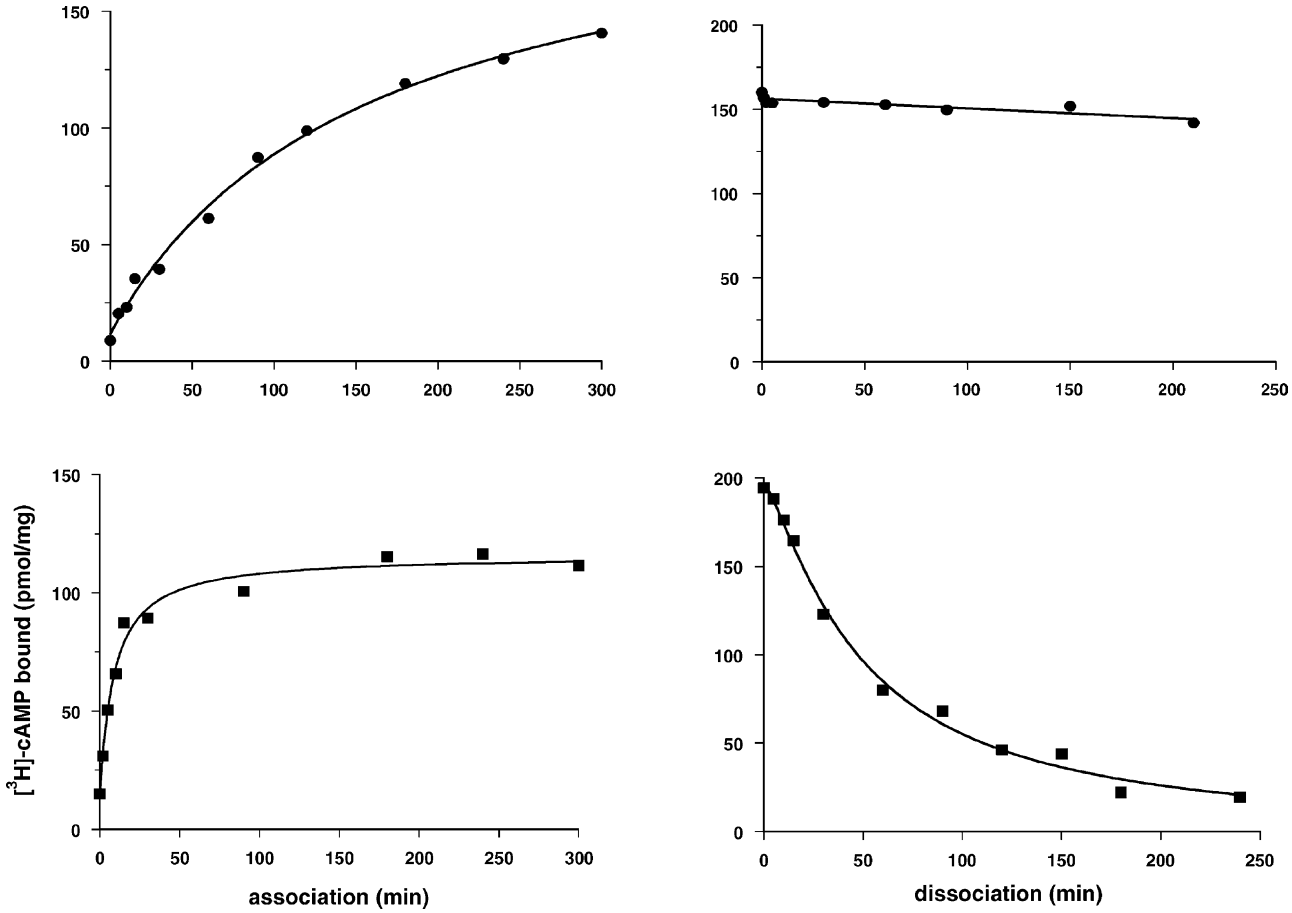

Fig. 1. Association and dissociation kinetics of specific [^{3}H]cAMP binding to AdoHcyase at 0 and 20°. AdoHcyase was incubated with [^{3}H]cAMP (25 nM) for various time intervals at different temperatures. Nonspecific binding was 20% of total binding. Data represent the means of one experiment performed in duplicate and are representative of three separate experiments.

However, at 20° the equilibrium of 25 nM [^{3}H]adenosine was reached after 60 min (Fig. 1). The kinetic experiments demonstrated that binding of [^{3}H]cAMP to AdoHcyase was nearly completely reversible at 20°. The time-course of association followed a single exponential function with a k_{+1} value of 0.035/min/M. Dissociation rate constant of [^{3}H]cAMP, k_{-1}, initiated by the addition of 1 mM unlabeled cAMP was 0.0121/min. The K_d value calculated from these kinetic constants was 26.5 nM. Similar results were obtained when dissociation was assessed by infinite dilution.

Saturation experiments were performed by increasing concentrations of [^{3}H]cAMP up to 300 nM. Nonspecific binding increased linearly with increasing [^{3}H]cAMP concentrations and represented approximately 20–25% of total binding. Nonlinear regression (Fig. 2) and Scatchard analysis (Inset Fig. 2) of the binding isotherms indicated that a single population of binding sites was labeled. The equilibrium dissociation constant (K_d) was 23.1 ± 1.1 nM and the maximal number of binding sites (B_{max}) labeled by [^{3}H]cAMP was 116.6 ± 3.8 pmol/mg protein. The maximal binding revealed a mole/mole ratio of cAMP/AdoHcyase of 0.025.

5.2. Displacement of [^{3}H]cAMP and [^{3}H]adenosine by adenosine analogues

In order to characterize further the pharmacological properties of the cAMP and adenosine binding site, competition experiments for [^{3}H]cAMP and [^{3}H]adenosine binding to AdoHcyase were performed with endogenous adenosine nucleosides and nucleotides, AdoHcyase inhibitors and adenosine receptors agonists and antagonists. Table 1 shows the EC_{50} values of adenosine derivatives at the [^{3}H]cAMP and [^{3}H]adenosine binding site. Adenosine and cAMP displace each other with similar affinities of EC_{50} 57 nM vs. EC_{50} 65 nM. Also the affinities of the compound tested were similar for the binding site of adenosine and cAMP. The rank order of potency of adenosine analogues for both binding sites was identical (adenosine > 2′-deoxyadenosine > N^6-methyladenosine > NECA > cAMP > diadenosine-diphosphate > 3-deazaadenosine). For an additional characterization we have studied the affinities of adenine nucleotides. Affinities of between 2.5 μM and 11 μM were found for the adenine nucleotides studied for both [^{3}H]cAMP and [^{3}H]adenosine binding sites. Neither

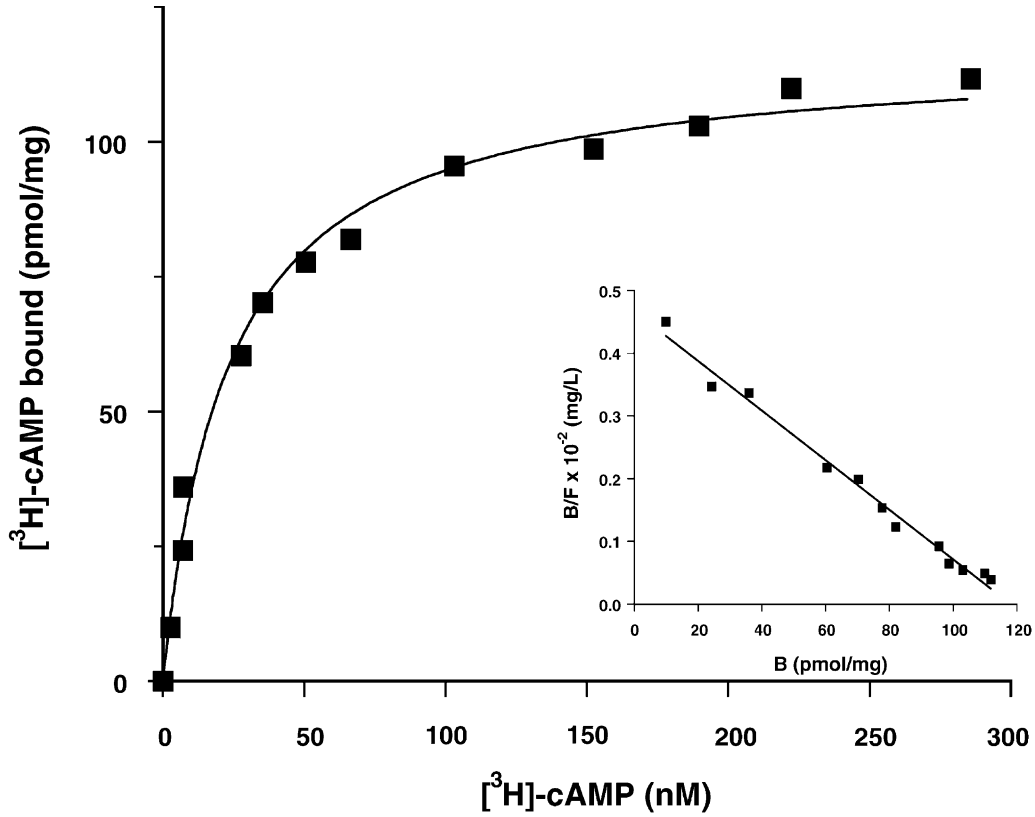

Fig. 2. Specific binding of [^{3}H]cAMP to purified AdoHcyase. AdoHcyase was incubated with increasing concentrations of [^{3}H]cAMP. Inset: transformation of the data according to Scatchard. Data were obtained from one experiment consisting of duplicate determinations and are representative of five independent experiments.

Table 1
Affinities of adenosine analogues for the [^{3}H]cAMP and [^{3}H]adenosine binding site on AdoHcyase

	EC_{50} [^{3}H]cAMP [M]	EC_{50} [^{3}H]adenosine [M]
Adenosine analogues		
Adenosine	65×10^{-9}	57×10^{-9}
cAMP	116×10^{-9}	104×10^{-9}
Inosine	16×10^{-6}	23×10^{-6}
2′-Deoxyadenosine	94×10^{-9}	92×10^{-9}
N^6-Methyladenosine	90×10^{-9}	95×10^{-9}
Diadenosine-diphosphate	139×10^{-9}	248×10^{-9}
Adenine	11×10^{-6}	9×10^{-6}
Hypoxanthine	$>10^{-4}$	$>10^{-4}$
AdoHcyase inhibitors		
Aristeromycin	5.6×10^{-6}	1.3×10^{-6}
Adenosine-2′, 3′-dialdehyde	9.3×10^{-6}	40×10^{-6}
3-Deazaadenosine	1.1×10^{-6}	3.2×10^{-6}
Adenine nucleotides		
ATP	11×10^{-6}	18×10^{-6}
AMP	2.5×10^{-6}	5.6×10^{-6}
A1-receptor agonists, antagonists		
NECA	80×10^{-9}	81×10^{-9}
2-Chloroadenosine	7×10^{-6}	5.8×10^{-6}
Theophylline	$>10^{-4}$	$>10^{-4}$
DPCPX	$>10^{-4}$	$>10^{-4}$

The EC_{50} values ± SEM are given. Each substance is tested in at least 7–9 different concentrations two to three times in duplicate.

[^{3}H]cAMP nor [^{3}H]adenosine could be displaced by hypoxanthine, theophylline, or DPCPX.

5.3. Titration of AdoHcyase by cAMP

As previously shown [5], 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 [5,20]. The spectrophotometric titration of the enzyme with adenosine and cAMP is shown in Fig. 3. The equilibrium constant calculated for adenosine was 245 ± 18 nM. The enzyme bound NAD^+ was completely transformed into the reduced form at an adenosine concentration of 10 μM. In contrast, cAMP was not able to reduce the enzyme bound NAD^+.

6. Discussion

It was previously shown that AdoHcyase from bovine kidney binds [^{3}H]adenosine with a high affinity [3]. In the present study we analyzed the binding of cAMP to purified AdoHcyase to compare it with adenosine to the enzyme.

AdoHcyase from bovine kidney exhibits one [^{3}H]cAMP binding site. In the saturation experiments we obtained a

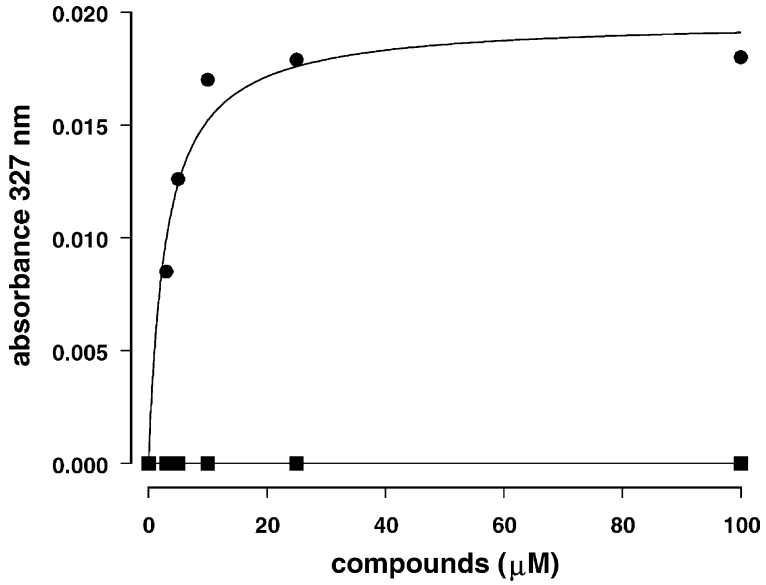

Fig. 3. Titration of AdoHcyase with adenosine and cAMP. The titration of 2.42 μM enzyme with adenosine (●) and cAMP (■) was monitored spectrophotometrically with an emission wavelength of 327 nm in 20 mM potassium phosphate pH 7.4 at room temperature to assess the reduction of NAD^+ to NADH. Data represent one experiment and are representative of three separate experiments.

high affinity dissociation constant of 23.1 ± 1.1 nM. This high affinity binding found in the saturation experiment was further analyzed. The K_d value determined from the rates of association and dissociation was 26.5 nM and is in good agreement with the K_d value obtained by equilibrium binding assay. Association and dissociation of [^{3}H]cAMP were found to be markedly temperature-dependent. At 0° only 10% of the bound [^{3}H]cAMP is displaced by an excess of unlabeled cAMP (1 mM), at 20° after 240 min a 90% dissociation is reached.

The high affinity dissociation constant found in our experiment is close to that value of 9 nM reported by Olsson [12] for an adenine analogue binding protein of the rabbit erythrocytes. Specific intracellular cAMP binding proteins, not associated with cAMP-dependent protein kinase were isolated from different species and organs with the evidence for two different binding sites for cAMP and adenosine [22–25]. In these studies, however, no high affinity binding site for adenosine was described.

Calculated from the B_{max} (116.6 pmol/mg) of cAMP binding we found a molar ratio of cAMP/AdoHcyase of 0.025/1. In our previous observation we found a molar ratio of 0.05/1 (adenosine/AdoHcyase) after binding of [^{3}H]adenosine to the high affinity binding site of AdoHcyase [3]. These different ratios do not argue against the assumption of one binding site for adenosine and cAMP since adenosine induces the reduction of the tightly bound NAD^+ to NADH resulting in a further increase of high adenosine binding sites [6,16,27]. However, cAMP does not transform the tightly bound NAD^+ to NADH (see Fig. 3). Since only 5% of our purified AdoHcyase binds [^{3}H]adenosine with this high affinity we measured the enzyme bound NAD^+/NADH fraction of AdoHcyase. Purified AdoHcyase from bovine kidney contains approximately 92–94% NAD^+ and 6–8% NADH. This finding together with the fact that NADH-AdoHcyase binds adenosine with high affinity and capacity [26,28] strongly favours the conclusion that [^{3}H]cAMP and [^{3}H]adenosine bind with high affinity at an allosteric and not at the catalytic site of AdoHcyase. The fact that adenosine and cAMP displace each other with similar affinities and all other purine compounds investigated displace cAMP and adenosine with equal potencies strongly favors the conclusion that the binding site for nanomolar concentrations of cAMP and adenosine at the AdoHcyase appears to be identical.

Since cAMP does not contains a 3′-OH group at the ribose it is not able to transform the tightly bound NAD^+ to NADH according to the catalytic mechanism of AdoHcyase [4,28]. In contrast to adenosine, cAMP can enhance the activity of AdoHcyase by 30% at 5 μM, while it is assayed in the direction of hydrolysis [15]. This might indicate that even at higher concentrations cAMP does not interact with the catalytic site of AdoHcyase.

With respect to the physiological importance of cAMP binding site at AdoHcyase one can assume that cAMP at ambient intracellular concentrations between 200 and 800 nM can compete with adenosine at the high affinity binding site. This competition would not result in a further inhibition of the enzyme activity, since cAMP does not reduce the enzyme bound NAD^+ to NADH.

Acknowledgments

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References

[1] Ueland PM. Pharmacological and biochemical aspects of S-adenosylhomocysteine and S-adenosylhomocysteine hydrolase. Pharmacol Rev 1982;34:223–53.

[2] de la Haba G, Cantoni GL. The enzymatic synthesis of S-adenosyl-L-homocysteine from adenosine and homocysteine. J Biol Chem 1959;234:606–8.

[3] Kloor D, Fuchs S, Kurz J, Faust B, Osswald H. S-Adenosylhomocysteine-hydrolase from bovine kidney: enzymatic and binding properties. Kidney Blood Press Res 1996;19:100–8.

[4] Palmer JL, Abeles RH. Mechanism of action of S-adenosylhomocysteinase. J Biol Chem 1979;254:1217–26.

[5] Kloor D, Fuchs S, Petroktistis F, Delabar U, Mühlbauer B, Quast U, Osswald H. Effects of ions on adenosine binding and enzyme activity of purified S-adenosylhomocysteine hydrolase from bovine kidney. Biochem Pharmacol 1998;56:1493–6.

[6] Kloor D, Osswald H. Control of enzyme activity and adenosine binding by the cofactor NAD^+/NADH of S-adenosylhomocysteine hydrolase. Drug Develop Res 2000;50:84.

[7] van Ments-Cohen M, Genieser HG, Jastorff B, van Hasastert PJM, Schaap P. Kinetics and nucleotide specificity of a surface cAMP binding site in *Dictyostelium discoideum*, which is not down-regulated by cAMP. FEMS Microbiol Lett 1991;66:9–14.

[8] Peters DJM, Bominaar AA, Snaar-Jagalska BE, Brandt R, van Hasastert PJM, Ceccarelli A, Williams JG, Schaap P. Selective induction of gene expression and second-messenger accumulation in *Dictyostelium discoideum* by the partial chemotactic antagonist 8-p-chlorophenylthioadenosine 3′5′-cyclic monophosphate. Proc Natl Acad Sci USA 1991;88:9219–23.

[9] Dubey RK, Gillespie DG, Zacharia LC, Mi Z, Jackson EK. A_{2B} receptors mediate the antimitogenic effects of adenosine in cardiac fibroblasts. Hypertension 2001;37:716–21.

[10] Hurta RA. S-Adenosylmethionine decarboxylase gene expression is regulated by cAMP signal transduction pathway in H-ras transformed fibrosarcoma cells capable of malignant progression. J Cell Biochem 2001;26:209–21.

[11] Døskeland SO, Øgreid D. Binding proteins for cyclic AMP in mammalian tissues. Int J Biochem 1981;13:1–19.

[12] Olsson RA. Ligand binding to the adenine analogue binding protein of the rabbit erythrocyte. Biochemistry 1978;17:367–75.

[13] Sæbo J, Ueland PM. A study on the sequestration of adenosine and its conversion to adenine by the cyclic AMP-adenosine binding protein S-adenosyl-homocysteinase from mouse liver. Biochim Biophys Acta 1979;587:333–40.

[14] Hershfield MS, Kredich NM. S-Adenosylhomocysteine hydrolase is an adenosine-binding protein: a target for adenosine toxicity. Science 1978;202:757–60.

[15] Danielyan L, Kloor D, Fuchs S, Osswald H. Binding of cAMP to a preparation of S-adenosylhomocysteine hydrolase from bovine kidney and influence of cAMP on enzyme activity. Biol Chem 1997;378: S147.

[16] Zimmerman TP, Schmitges CJ, Wolberg G, Deeprose RD, Duncan GS, Cuatrecasas P, Elion G. Modulation of cyclic AMP metabolism by S-adenosylhomocysteine hydrolase and S-3-deazaadenosylhomocysteine in mouse lymphocytes. Proc Natl Acad Sci USA 1980;77: 5543–639.

[17] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal Biochem 1976;72:248–54.

[18] Bruns RF, Lawson-Wendling K, Pugsley TA. A rapid filtration assay for soluble receptor using polyethylenimine-treated filters. Anal Biochem 1983;132:74–81.

[19] Gomi T, Takata Y, Fujioka M. Rat liver S-adenosylhomocyteinase. Spectrophotometric study of coenzyme binding. Biochim Biophys Acta 1989;994:172–9.

[20] Matuszewska B, Borchardt RT. The role of nicotinamide adenine dinucleotide in the inhibition of bovine liver S-adenosylhomocysteine hydrolase by neplanocin A. J Biol Chem 1987;262:265–8.

[21] Munson PJ, Rodbard D. LIGAND: a versatile computerized approach for the characterization of ligand binding systems. Anal Biochem 1980;107:220–39.

[22] Ueland PM, Døskeland SO. An adenosine 3′,5′-monophosphate-adenosine binding protein from mouse liver. Purification and partial characterisation. J Biol Chem 1977;252:677–86.

[23] Ueland PM, Døskeland SO. An adenosine 3′,5′-monophosphate-adenosine binding protein from mouse liver. A study on its interaction with adenosine-3′,5′-monophosphate and adenosine. J Biol Chem 1978;253:1667–76.

[24] Yuh KC, Tao M. Purification and characterization of adenosine–adenosine cyclic 3′,5′-monophosphate binding protein factors from rabbit erythrocytes. Biochemistry 1974;13:5220–6.

[25] Sudgen PH, Carbin JD. Adenosine 3′,5′-cyclic monophosphate-binding proteins in bovine and rat tissues. Biochem J 1976;159:423–37.

[26] Kloor D, Yao K, Delabar U, Osswald H. Simple and sensitive assay for measurement of adenosine using reduced S-adenosylhomocysteine hydrolase. Clin Chem 2000;46:537–42.

[27] Abeles RH, Fish S, Lapinskas B. S-Adenosylhomocysteinase: mechanism of inactivation by 2′-deoxyadenosine and interaction with other nucleotides. Biochemistry 1982;21:5557–62.

[28] Porter DJT, Boyd FL. Reduceced S-adenosylhomocysteine hydrolase. Kinetics and thermodynamics for binding of 3′-ketoadenosine, adenosine, and adenine. J Biol Chem 1992;267:3205–13.