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ADENOSYLHOMOCYSTEINASE:ADENOSINE COMPLEX +

Hieronim Jakubowski and Andrzej Guranowski

Institute of Biochemistry, Academy of Agriculture,
Wołyńska 35, 60-637 Poznań, Poland

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SUMMARY: Adenosylhomocysteinase from yellow lupin seeds forms a specific complex with adenosine. The complex can be isolated either by nonequilibrium or equilibrium gel filtration. It is also adsorbed on nitrocellulose disks. Dissociation constant of the complex determined by nitrocellulose filter assay is 5×10^{-8}M.

INTRODUCTION

Adenosylhomocysteinase (EC 3.3.1.1) which catalyzes the following reaction:

S-Adenosyl-L-homocysteine + $H_2O \rightleftharpoons$ Adenosine + L-Homocysteine

was first found in rat liver by de la Haba and Cantoni over twenty years ago [1]. In the last years interest in this enzyme renewed because several observations demonstrated that adenosylhomocysteine and its analogs are potent inhibitors of a number of methyltransferases utilizing S-adenosylmethionine as the methyl donor [2-6]. Adenosylhomocysteinase has been found in all eukaryotes examined [7] and recently the enzyme was obtained almost simultaneously and independently in a homogeneous state from both animal [8,9] and plant [10] sources. The reaction catalyzed by

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adenosylhomocysteinase seems to be an attractive model for studying mechanisms of carbon-sulfur bond formation. Palmer and Abeles (8) demonstrated tightly bound NAD in the beef liver adenosylhomocysteinase and proposed a mechanism for the reversible hydrolysis of S-adenosyl-L-homocysteine involving oxidation of position 3' of adenosine followed by α-β elimination of L-homocysteine.

As an approach to study the mechanism of action of plant adenosylhomocysteinase, we attempted to isolate the enzyme-substrate complexes. In this report we describe the properties of stable adenosylhomocysteinase:adenosine complex which may be retained by nitrocellulose disks and isolated by nonequilibrium and equilibrium gel filtration.

MATERIALS AND METHODS

Homogeneous adenosylhomocysteinase from yellow lupin seeds and chromatographically pure [U-^{14}C] adenosine (500 Ci/mol) were obtained as described previously (10). [U-^{14}C] Uridine was from C.E.A. Saclay, France. Sephadex G-50 and G-50 Fine were from Pharmacia, Sweden. Sources of the other chemicals used were given previously (10). Cellulose nitrate filters (24 mm in diameter, type BA 85) were from Schleicher & Schüll, F.R.G.. Separation of adenosine and S-adenosyl-L-homocysteine was performed according to the method of Poulton and Butt (11). Nitrocellulose filter assay was carried out as described by Yarus and Berg (12). The disks were presoaked in solution used for incubation and washing and placed on filter holder. After the enzyme had been mixed with ligand the samples were kept on ice for 1 min and applied under moderate suction on the filters. Next the filters were washed four times with 1-ml portions of appropriate ice cold solution, dried and counted in a scintillation counter. Blanks of incubation without enzyme were subtracted from all assay values. Details of nonequilibrium and equilibrium gel filtration are in

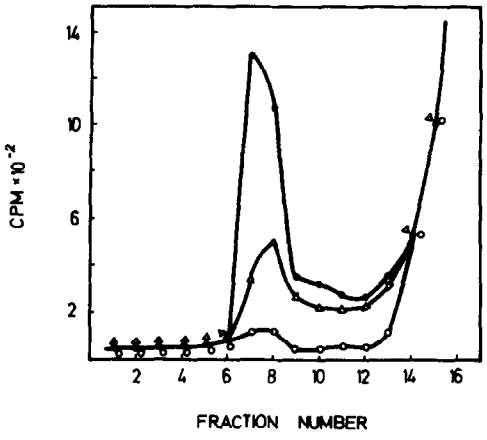


Fig.1. Isolation of adenosylhomocysteinase:adenosine complex on Sephadex G-50 column.
The complex was prepared by mixing 320 pmoles of adenosylhomocysteinase in a volume of 100 µl solution with 2.5 mM mercaptoethanol, 50 mM buffer, potassium phosphate, pH 6.8 (●), (○) or Tris/HCl, pH 8.3, (△) and either 10 µM [^{14}C]adenosine (●), (△) or 10 µM [^{14}C]adenosine + 5 mM DL-homocysteine (○). After 1 min at 0°C the mixture was applied onto Sephadex G-50 column (1 x 9 cm) previously equilibrated with appropriate buffer and eluted with the same buffer by gravity at 4°C. 0.44 ml fractions were collected and 100 µl aliquots of each fraction spotted on filter paper disks, oven-dried and counted on Beckman LS-100 scintillation counter.

the figure legends. Protein concentration was determined by the turbidimetric tannin method (13).

RESULTS AND DISCUSSION

Isolation of adenosylhomocysteinase:adenosine complex by gel filtration

When the mixture containing [U-^{14}C]adenosine and substrate amounts of homogeneous adenosylhomocysteinase was passed through small Sephadex G-50 column, some of the radioactivity (approximately 2.5%) emerged in the break-through fractions (Fig.1, trace ●). After homocysteine (final concentration

5 mM) was added to the fractions and the aliquots immediately analyzed by thin layer chromatography, radioactive S-adenosylhomocysteine was detected. This indicates that the break-through Sephadex fractions contained adenosylhomocysteinase:adenosine complex which was enzymatically active. The same amounts of the complex were isolated in the potassium phosphate, pH 6.8 and 8.2, while 3 times less complex was achieved in Tris/HCl buffer, pH 8.3, (Fig.1, trace Δ). In another experiment, when a mixture containing adenosylhomocysteinase and both substrates, [^{14}C]adenosine and homocysteine, was filtrated through the Sephadex column, only traces of radioactivity were found in break-through fractions (Fig.1, trace O). Since in the presence of 5 mM homocysteine (under these conditions) 95% of [^{14}C]adenosine was transformed into [^{14}C]adenosylhomocysteine before gel filtration, it appears that adenosylhomocysteinase does not form a stable complex with S-adenosylhomocysteine.

Adsorption of adenosylhomocysteinase:adenosine complex on nitrocellulose disks

The adenosylhomocysteinase:[^{14}C]adenosine complex is retained on nitrocellulose disks. As shown in Fig.2, the amount of the complex is proportional to the amount of adenosylhomocysteinase over range 0-90 pmoles. Retention is an attribute of native adenosylhomocysteinase, since if [^{14}C] adenosine was mixed with bovine serum albumin, heat-inactivated enzyme or the enzyme preincubated with the irreversible inhibitor, p-hydroxymercuribenzoate (10), no radioactivity over the level of the controls which were run without the active enzyme was adsorbed. It is noticeable that the thiol group(s) in the lupin enzyme is (are) essential

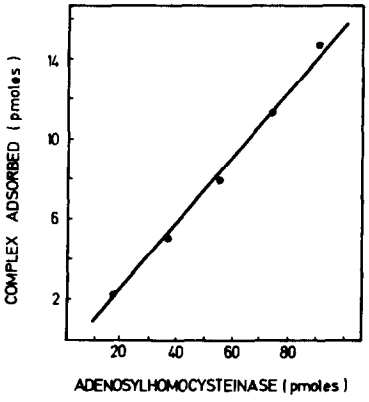


Fig.2. Retention of adenosylhomocysteinase:adenosine complex by nitrocellulose filters.
The assays were carried out as described in Materials and Methods. The indicated amounts of enzyme were applied on the filters in 50 µl portions of solution containing 50 mM potassium phosphate, pH 6.8, 2.5 mM mercaptoethanol, and 2.5 µM [^{14}C] adenosine.

for adenosine binding. Nor was any radioactivity retained on the filters when either [^{14}C] uridine or [^{14}C]ATP was incubated with the adenosylhomocysteinase.
While during gel filtration in the potassium phosphate buffers the same amounts of the adenosylhomocysteinase: adenosine complex were obtained both at pH 6.8 and 8.2, retention of the complex on nitrocellulose filters was markedly affected by pH of the buffer used for washing (Fig.3). As already noticed by Yarus and Berg (12), who studied the adsorption of another enzyme-ligand systems on nitrocellulose disks, the composition of the wash fluid influences the efficiency of complex binding. In contrast with isoleucyl-tRNA synthetase:tRNA complex which was best retained at pH 5-5.5, the efficiency of retention of adenosylhomocysteinase:adenosine complex was highest at

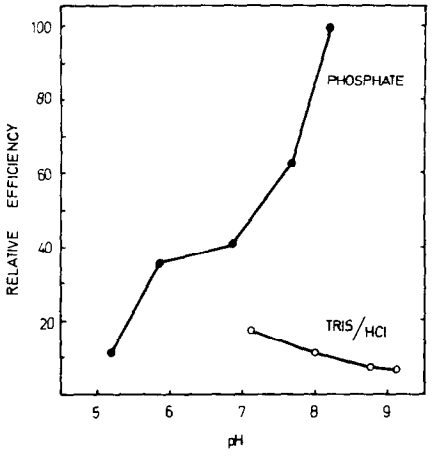
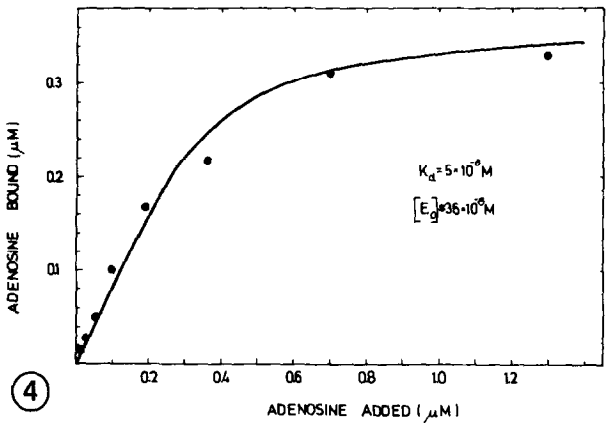


Fig.3. Effect of pH of reaction mixture and wash fluid on relative efficiency of adenosylhomocysteinase:adenosine complex detection.
Reaction mixture (50 µl) contained 2.5 µM [^{14}C]adenosine, 2.5 mM mercaptoethanol and 50 pmoles of adenosylhomocysteinase. The pH of reaction mixture and wash fluid varied together by changing the composition of buffer.

pH 8.2. The radioactive adenosylhomocysteinase:adenosine complex disappeared from disks when they were washed with distilled water. The complex obtained by gel filtration was stable for at least 2 hours both at 0° and 25°C, as checked by nitrocellulose filtration assay. [^{14}C]Adenosine does not bind covalently to adenosylhomocysteinase since no [^{14}C]label remains in the 10% trichloroacetic acid precipitate collected on the filters.

Titration of adenosylhomocysteinase with [^{14}C]adenosine by the nitrocellulose filter assay

The accumulation of enzyme-bound adenosine as a function of [^{14}C]adenosine concentration was measured by trapping the [^{14}C] labeled complex on nitrocellulose disks.



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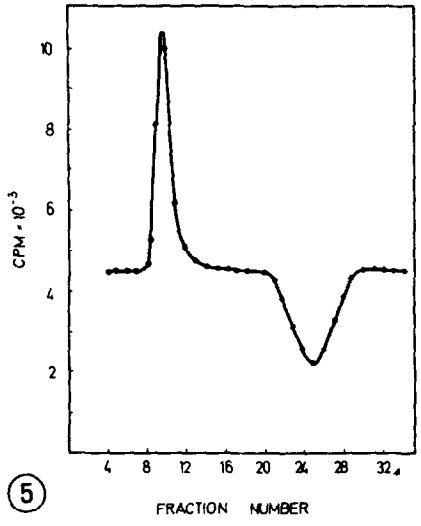

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Fig.4. Titration of adenosylhomocysteinase with [^{14}C] adenosine by the nitrocellulose filter assay.
Each sample (50 μl) applied on the filter contained 50 mM potassium phosphate, pH 8.2, 2.5 mM mercaptoethanol, $[E_o] = 3.6 \times 10^{-7}$ M adenosylhomocysteinase and varied concentrations of [^{14}C] adenosine (880 counts x min^{-1} x $pmol^{-1}$). The efficiency of retention was 25%. Experimental points: (●) ; solid line = theoretical calculated curve.

$$y = \frac{1}{2}\left([S_o] + [E_o] + K_d - \sqrt{([S_o] + [E_o] + K_d)^2 - 4[E_o][S_o]}\right), \quad (14).$$

Fig.5. Equilibrium gel filtration of lupin adenosylhomocysteinase.
Fifty pmoles of adenosylhomocysteinase in 10 μl was applied on the Sephadex G-50 Fine column (0.6 x 4 cm) previously equilibrated with the solution containing 50 mM potassium phosphate, pH 8.2, 2.5 mM mercaptoethanol and 0.1 μM [^{14}C] adenosine and eluted with the same solution. Two-drop-fractions (60 μl) were collected on paper disks, oven-dried and counted.

The experimental points fit a theoretical curve (14) which corresponds to a stoichiometry of 1 mole of [^{14}C] adenosine per mole of enzyme and to a dissociation constant of 5×10^{-8} M (see Fig.4). Concentration of adenosylhomocysteinase

was calculated from its molecular weight of 110 000 daltons and protein concentration.

Equilibrium gel filtration

The adenosylhomocysteinase:adenosine complex was also detected by equilibrium gel filtration (Fig.5). At 10^{-7}M [^{14}C] adenosine in the equilibrating buffer about 25% of the enzyme applied on the column was complexed with adenosine. This figure is compatible with the K_d value 5×10^{-8}M determined by nitrocellulose filter assay (see above).

Adenosylhomocysteinase forms an extremely tight complex, $K_d = 5 \times 10^{-8}$M, with one of its substrates, adenosine. Taking into account the *in vivo* concentration of the enzyme, which may be calculated as 1-3 μM (15), and the concentration of adenosine in plant tissue, which may be assessed as about 40 μM (16), one might conclude that *in vivo* adenosylhomocysteinase occurs in the form of a complex with adenosine. There seems to be no correlation between the enzyme subunit structure (α_2) and the stoichiometry of adenosine binding. Thus, adenosylhomocysteinase appears to exhibit half-of-the sites-reactivity (17) under our experimental conditions.

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