A SPECIFIC ADENOSINE PHOSPHORYLASE, DISTINCT FROM PURINE NUCLEOSIDE PHOSPHORYLASE

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1. Introduction

Several enzymes, catalyzing the reversible phosphorolysis of purine nucleosides have been described in eucaryotic cells and in micro-organisms [1–6]. The best known is the 'purine nucleoside phosphorylase' (EC 2.4.2.1), acting on the nucleosides of hypoxanthine and guanine. Adenosine is not used as substrate by this enzyme [6–8]. Phosphorolysis of adenosine has been reported in *Salmonella typhimurium* [9], where a single enzyme protein appears to act on inosine, guanosine and adenosine. Adenine was found to be substrate for purine nucleoside phosphorylase of four mammalian sources, but its unfavourable kinetic parameters with respect to those of hypoxanthine and guanine are against the role of adenine as a physiological substrate [10,11]. In *Mycoplasma* both adenosine and inosine phosphorolysis have been observed, but no attempt has been made to correlate the two activities to different proteins [12]. The separation of adenosine phosphorylase from the purine nucleoside phosphorylase has never been reported so far, even though Miech and Coll. have presented indirect evidence that in *Schistosoma Mansoni* worms adenosine phosphorylase activity is a separate entity from purine nucleoside phosphorylase: this conclusion is based on differences in the pH–activity curves and studies with product and nucleoside analogues inhibitors [13].

The data presented in this paper give the first direct evidence that at least in *B. subtilis* the phosphorolysis of adenosine and that of inosine and guanosine are catalyzed by distinct enzyme proteins, which can be partly separated by ammonium sulphate precipitation and Sephadex G-100 gel filtration.

2. Materials and methods

Nucleosides and bases were obtained from Sigma Chemical Co. or from Boehringer und Soehne. Adenosine deaminase from intestinal mucosa was obtained from Boehringer und Soehne.

Preparation of crude extracts: Cell-free extracts of vegetative forms and free spores of *B. subtilis* were prepared as previously described [14]. Crude extracts were subjected to ammonium sulphate fractionation between 0–20, 20–30, 30–40, 50–60, 60–70, 70–80% saturation. The precipitates were dissolved in a minimal volume of Tris-Cl buffer, 0.05 M, pH 7.3 and directly used to measure enzyme activities. Sephadex G-100 gel filtration of several fractions, presenting different ratios of adenosine phosphorylase activity to purine nucleoside phosphorylase activity, was accomplished as described under the legends to figs.1 and 2.

Enzyme assays: Purine nucleoside phosphorylase assay was carried out as previously described [15] using both inosine and guanosine as substrates.

Adenosine deaminase (EC 3.5.4.4), xanthine oxidase (EC 1.2.3.2), guanase (EC 3.5.4.3) and adenase (EC 3.5.4.2) were measured according to Kalckar [16], and were always found absent in crude extracts, in fractions precipitated with ammonium sulphate, and in column eluates.

Adenosine phosphorylase was assayed spectrophoto-

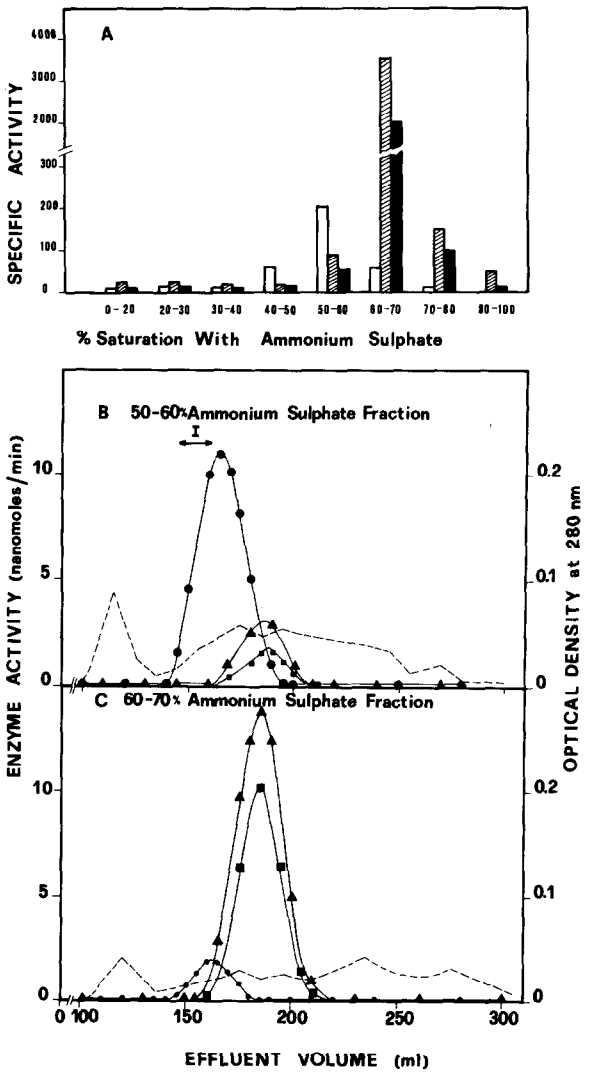

Fig.1. Separation of adenosine phosphorylase and purine nucleoside phosphorylase of *Bacillus subtilis* spore extracts. (A) Specific activities (nanomoles of substrate transformed/min/mg of protein) of adenosine phosphorylase (□), inosine phosphorylase (▨) and guanosine phosphorylase (■) of different fractions obtained by ammonium sulphate fractionation of spore extracts. (B) and (C) Elution patterns of adenosine phosphorylase and purine nucleoside phosphorylase of *Bacillus subtilis* spore extracts. 8 ml of the fraction precipitated between 50 and 60% saturation (B) and 8 ml of the fraction precipitated between 60 and 70% saturation (C), each containing approx. 4.5 mg protein/ml were eluted from Sephadex G-100 columns (3 ×90 cm) equilibrated with 0.25 M Tris-Cl buffer, pH 7.3, at a flow rate of 15 ml/h. Fractions of 5 ml were collected. 0.2 ml of each fraction was used to measure enzyme activities. The velocities are expressed as nanomoles of adenosine (adenosine phosphorylase, assay A) (●——●) and hypoxanthine (▲——▲) or guanine (■——■) (purine nucleoside phosphorylase) formed per minute. The fraction indicated by I (pool I) were used to measure the K_M and V_{max} values.

metrically using adenine and ribose 1-phosphate as substrates. The formation of adenosine was measured in a coupled reaction with adenosine deaminase [13].

The detailed procedure was as follows: 0.6 ml of 0.25 M Tris-Cl buffer, pH 7.3, was pipetted into one cuvette, followed by different amounts of aqueous solutions of adenine and ribose 1-phosphate, each contained in a maximal volume of 0.1 ml, 2 μg of adenosine deaminase, and enough water to bring the volume to 0.95 ml. Finally, 0.05 ml of enzyme preparation was added. The mixture was rapidly mixed, and the change in optical density at 265 nm was followed against a reference cuvette, in which adenine was substituted with water. This procedure will be referred to as 'assay A'. The molar spectral change was taken as 6500 O.D. units, the difference between adenine and inosine at pH 7.3. At concentrations of adenosine deaminase higher than 1 μg per reaction mixture, no lag period was observed, and the rate of the decrease in optical density remained constant, with increasing concentration of adenosine deaminase. The velocity of the reaction is therefore determined by the rate at which adenosine is formed.

In the absence of adenosine phosphorylase, the absorbance at 265 nm remained constant, showing the absence of adenosine phosphorylase activity in the adenosine deaminase used as auxiliary enzyme. Similarly, when adenosine phosphorylase and adenosine deaminase were added after adenine, no change in optical density was observed, and the reaction became dependent on the addition of ribose 1-phosphate.

The velocity of the reaction was strictly proportional to the amount of protein up to higher than 0.200 absorbance units/min. All studies reported here were conducted at rates less than 0.080 absorbance units/min.

In contrast with adenosine phosphorylase activity from *Schistosoma Mansoni* [13] no inhibition by Tris-Cl was observed.

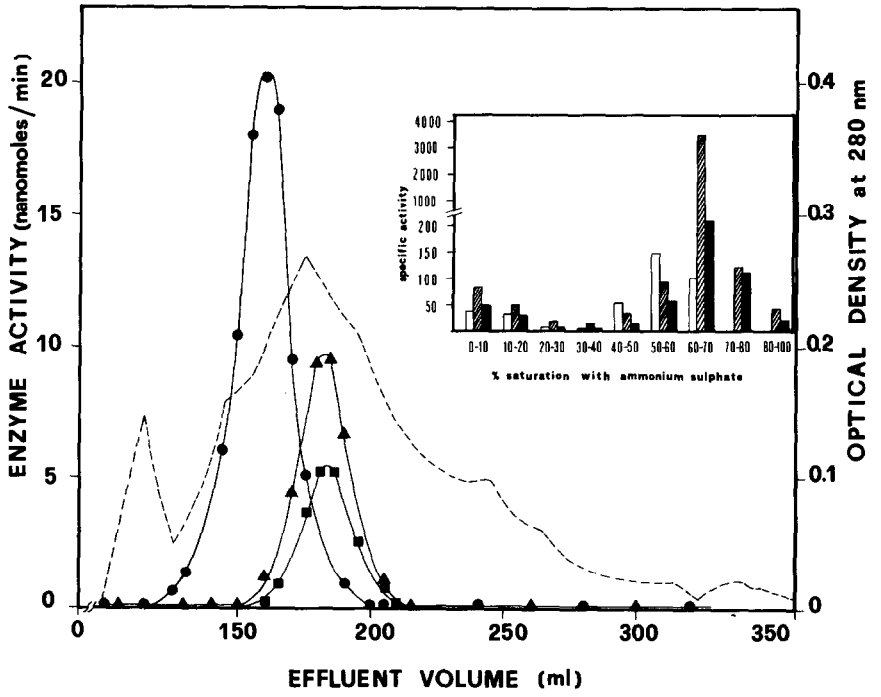

Fig.2. Elution patterns of adenosine phosphorylase and purine nucleoside phosphorylase of *Bacillus subtilis* vegetative forms. 8 ml of the fraction precipitated between 50 and 60% ammonium sulphate saturation was subjected to the same procedure described in fig.1. The velocities are expressed as nanomoles of adenosine (adenosine phosphorylase, assay A) (●——●) and hypoxanthine (▲——▲) or guanine (■——■) (purine nucleoside phosphorylase) formed per min. The inset shows the specific activities (nanomoles of substrate transformed/min/mg of protein) of adenosine phosphorylase (□), inosine phosphorylase (▨) and guanosine phosphorylase (■) of different fractions obtained by ammonium sulphate fractionation.

A less sensitive spectrophotometric assay was also used ('Assay B'), based on the phosphate dependent rate of adenosine disappearance by measuring the fall in absorbance at 275 nm, which accompanies the conversion of adenosine (molar absorbance coefficient 6480) to adenine (molar absorbance coefficient 4240), at pH 7.3. The standard reaction mixture contained 0.6 ml 0.1 M potassium phosphate buffer, pH 7.3, varying amounts of aqueous solutions of adenosine, contained in a maximal volume of 0.08 ml, enzyme preparation, and water to a final volume of 1 ml. The reaction was monitored by a D.B. recording Acta III spectrophotometer, against a reference cuvet, in which adenosine was substituted by water. No reaction occurred when phosphate buffer was substituted with Tris-Cl buffer. This method could not be used with crude extracts, but was found suitable to measure kinetic parameters for adenosine and inorganic phosphate with Sephadex G-100 columns eluates (pool 1 of fig.1).

3. Results and discussion

Maximal adenosine phosphorylase activity was found in the fraction precipitated between 50 and 60% ammonium sulphate saturation, both in vegetative forms and in spores of *B. subtilis* while maximal inosine-guanosine phosphorylase activity was found in the fraction precipitated between 60 and 70% saturation (fig.1A and inset of fig.2). Further separation of adenosine phosphorylase and purine nucleoside phosphorylase was achieved by the use of Sephadex G-100 gel filtration, leading to the appearance of two distinct peaks, with adenosine and inosine-guanosine phosphorylase activity respectively,

both in spores (fig.1B and C) and in vegetative forms (fig.2).

From double reciprocal plots the following K_M and V_{max} values were calculated: adenosine (4.5×10^{-5} M; 1550 nmol/min/mg); inorganic phosphate (7.5×10^{-3} M; 1680 nmol/min/mg); adenine (3.05×10^{-5} M; 1580 nmol/min/mg); ribose 1-phosphate (1.35×10^{-4} M; 1538 nmol/min/mg). The values for one substrate were obtained at saturating concentration of the other.

Equilibrium constant: a reaction mixture, containing 0.1 M Tris-Cl buffer, pH 7.3, 0.055 mM adenosine, 7 mM K_2HPO_4 and 25 μg of enzyme protein (peak 1 of fig.2) in a final volume of 7 ml was divided into one aliquot of 1 ml and two aliquots of 3 ml each. The 1-ml aliquot was monitored at 275 nm (assay B) at 37°C. One of the two 3-ml aliquots was incubated at 37°C in a sealed tube. After 1 h, when equilibrium was reached, the tube was heated 5 min at 75°C, to inactivate adenosine phosphorylase which proved in preliminary experiments to be extremely sensitive to heat, and cooled to 37°C. The other 3-ml aliquot was heated and cooled as above at zero time. The amount of adenosine in the two 3-ml aliquots, corresponding to adenosine at equilibrium and to adenosine at zero time was determined spectrophotometrically at 37°C, after addition of 0.4 μg of commercial adenosine deaminase, by measuring the total absorbance change which accompanies the conversion of adenosine to inosine; the molar spectral change at 265 nm was taken as 8100. It must be pointed out that the concentration of adenosine determined at zero time in these conditions was exactly coincident to that of the original reaction mixture.

The equilibrium constant:

$$K_{eq} = \frac{[Ade]_{eq} \cdot [Rib\text{-}1\text{-}P]_{eq}}{[Ado]_{eq} \cdot [P_i]_{eq}}$$

was calculated by assuming,

$$[Ade]_{eq} = [Rib\text{-}1\text{-}P]_{eq} = [Ado]_{zero\ time} - [Ade]_{eq}$$

and

$$[P_i]_{eq} = [P_i]_{zero\ time} - [Ade]_{eq}$$

The values of two determinations were 6.02×10^{-3} and 6.60×10^{-3}. Other determinations were carried out at different ratios of $Ado_{initial}$ and $P_{i\ initial}$ at 0.1 mM adenosine and 3.2 mM K_2HPO_4, at 0.2 mM $P_{i\ (initial)}$ adenosine and 0.08 mM P_i, and at 0.1 mM adenosine and 1.6 mM P_i values of 4.8×10^{-3}, 5.8×10^{-3} and 5.9×10^{-3} respectively were obtained. The equilibrium of the reaction is therefore towards adenosine formation. For other purine nucleoside phosphorylase reactions, the equilibrium is also towards nucleoside formation [17,18].

Our results show that in *Bacillus subtilis* adenosine phosphorylase is clearly distinct from the so called purine nucleoside phosphorylase, acting on guanosine and inosine. The two enzyme activities can be clearly separated by ammonium sulphate fractionation and by filtration on Sephadex G-100.

Evidence that the cleavage of the *N*-glycosidic bond of adenosine is phosphorolytic stands on the observations that adenosine is not cleaved in the absence of inorganic phosphate and that adenosine formation has an absolute requirement of ribose 1-phosphate.

The equilibrium constant of the reaction strongly favors adenosine formation. However, the physiological role of the enzyme remains to be estabilished. We postulate that the enzyme is involved in a 'purine salvage pathway', via the successive action of an adenosine kinase or of an AMP pyrophosphorylase.

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