SOME REGULATORY PROPERTIES OF PURINE NUCLEOSIDE PHOSPHORYLASE OF *BACILLUS CEREUS*

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1. Introduction

In addition to the *de novo* synthesis of nucleotides [1, 2] other routes for the formation of these compounds are known. They include the so-called "salvage pathways" which are employed to utilize exogenous sources of preformed bases or nucleosides [3]. The occurrence of the enzymes of the "salvage pathways" seems to be universal [4]. As their importance has become evident, some studies have been carried out to investigate the regulatory mechanism of these metabolic routes [5, 6].

The results presented in this report show that, in extracts of *Bacillus cereus* AICB 8122 vegetative cells, adenosine is readily converted to hypoxanthine by the combined action of adenosine deaminase and inosine phosphorylase; the base is then transformed into IMP, the common precursor of AMP and GMP, in the presence of 5-phosphoribosyl-1-pyrophosphate (PRPP). This "salvage pathway" is regulated by the intracellular concentration of nucleotides through feed-back inhibition of the reaction catalyzed by inosine phosphorylase.

In *B. cereus* spores and vegetative cells an enzyme activity catalyzing the deamination of adenosine was originally found by Powell and Hunter [7] and by Powell and Strange [8].

Inosine phosphorylase (EC 2.4.2.1), which is presumably the same as purine nucleoside phosphorylase, had been extracted from vegetative cells and spores of *B. cereus* var. *terminalis* and its physical and catalytic properties had been studied [9, 10]. However, the metabolic role and regulatory properties of *B. cereus* inosine phosphorylase have not been investigated.

2. Experimental

2.1. *Materials*

Nucleotides, nucleosides and bases were obtained either from C.F. Boehringer & Soehne (Mannheim, Germany), or from Sigma Chemical Company (St. Louis, Mo., USA). ApA, ApG and GpA were purchased from Zellstofffabrik (Mannheim, Germany). Type 1-A ribonuclease from bovine pancreas (5X crystallized) was obtained from Sigma Chemical Company. Lactate dehydrogenase from rabbit muscle and adenosine deaminase from calf intestinal mucosa were obtained from Boehringer & Soehne. Whale skeletal myoglobin was obtained from Seravac Laboratories (Maidenhead, England).

2.2. *Preparation of extracts*

Cultures and extracts of *B. cereus* AICB 8122 were prepared as previously described [11] to obtain the "supernatant fluid", which was used as such or after gel filtration through Sephadex G-100 according to Andrews [12]. Columns (3 X 90 cm) were equilibrated with 0.05 M Tris-Cl buffer pH 7.4; 2 ml

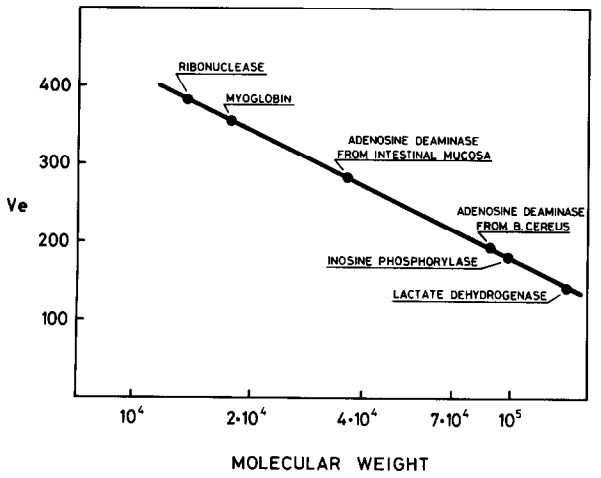

Fig. 1. Plot of elution volumes (V_e) against molecular weight for adenosine deaminase and inosine phosphorylase from *B. cereus* and other marker proteins on Sephadex G-100. Myoglobin, pancreatic ribonuclease, lactic dehydrogenase and adenosine deaminase from intestinal mucosa (approximately 3 mg of total protein) were dissolved in 2 ml of the crude extract of *B. cereus*. The solution was applied to the top of a Sephadex G-100 column (3 × 90 cm). Elution was carried out as described under "Experimental". Elution volumes for adenosine deaminase, lactic dehydrogenase and ribonuclease were estimated by assaying the effluents by the methods of Kalckar [14], Neilands [18] and Ipata and Felicioli [19], respectively. Elution volume for myoglobin was estimated at 407 nm.

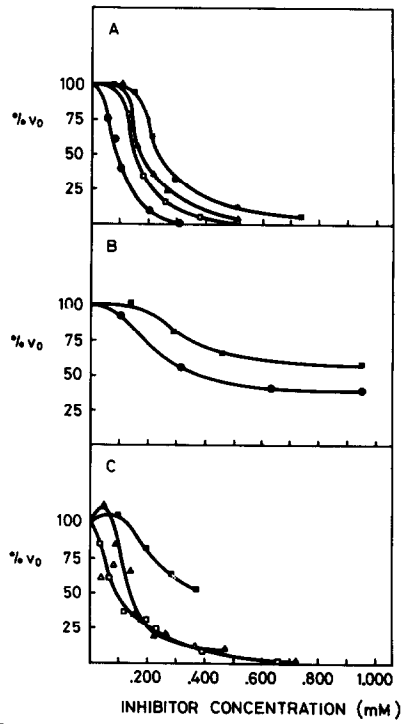

Fig. 2. Effect of varying concentrations of inhibitors on the initial velocity of inosine phosphorylase. The final inosine concentration was 0.5 mM. The velocity is expressed as % of the velocity in absence of inhibitors. A: ■, GMP; ▲, GTP; ●, XMP; □, GTP + GMP. B: ■, AMP; ●, ATP. C: ■, UMP; ▲, CMP; △, UTP; □, CTP.

portions of the crude extract were applied and eluted in 5 ml fractions with the same buffer at a flow rate of 18 ml/hr. The protein content was determined by the biuret method [13].

2.3. *Assay procedures*

Adenosine deaminase was assayed spectrophotometrically at 265 nm according to Kalckar [14]. The standard assay mixture contained 0.4 ml of 0.2 M potassium phosphate buffer pH 7.0, different amounts of an aqueous solution of adenosine contained in a maximal volume of 0.1 ml, enzyme preparation (Sephadex G-100 eluates) and water to a final volume of 1 ml. The activity of inosine phosphorylase was followed spectrophotometrically from the phosphate-dependent rate of inosine disappearance, by measuring the fall in absorbance at 280 nm which accompanies the conversion of inosine (molar absorbance coefficient 1850) to hypoxanthine (molar absorbance coefficient 729) at pH 7.0. The standard reaction mixture contained 0.6 ml of 0.1 M potassium phosphate buffer pH 7.0, different amounts of an aqueous solution of inosine contained in a maximal volume of 0.050 ml, enzyme preparation (G-100 eluates) and water to a final volume of 1 ml.

The activities of xanthine oxidase and guanase were assayed spectrophotometrically according to Kalckar [15] and were always found to be absent in *B. cereus* extracts.

3. Results and discussion

The products of the enzymic degradation of adenosine were identified by paper chromatography in the propanol-trichloroacetic acid–NH_3–water system described by Cerletti et al. [16]. Incubation

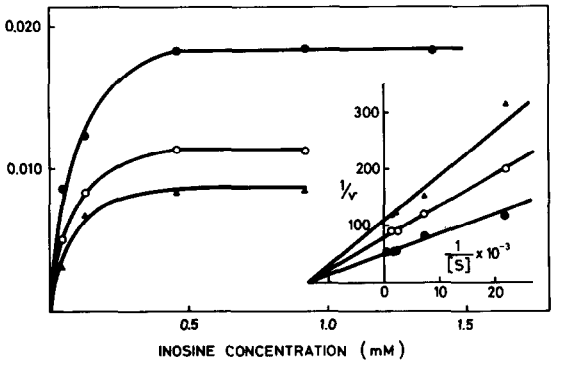

Fig. 3. Initial velocity plots of *B. cereus* inosine phosphorylase in the absence and in the presence of GMP. The inset shows the plots 1/v versus 1/[S]. The velocity is expressed as Δ absorbance per minute at 280 nm. •, no GMP; ○, GMP 0.30 mM; △, GMP 0.36 mM.

Table 1
Effect of various nucleotides on inosine phosphorylase activity.

Inhibitor	Concentration required for 50% inhibition (mM)	% inhibition at saturating inhibitor concentration (mM)	Hill coefficient for the inhibition curve
XMP	0.090	100 at 0.300	2.63
GTP	0.170	100 at 0.700	2.78
GMP	0.230	100 at 1.000	2.50
GTP + GMP	0.075 (each)	100 at 0.500	3.57
ATP	0.360	60 at 0.800	2.22
AMP	–	45 at 1.000	–
CTP	0.080	100 at 0.800	–
UTP	0.080	100 at 0.800	–
CMP	0.150	90 at 0.500	–
UMP	0.450	–	–

The reaction mixtures contained in a final volume of 1 ml 0.06 M potassium phosphate buffer pH 7.0, 0.5 mM inosine, different inhibitor concentrations and 0.5 mg protein from G-100 eluate. The reaction was started by addition of the enzyme preparation and the decrease in absorbance at 280 nm was recorded against a reference cuvette in which the substrate was replaced by water.

mixtures consisted of 1 ml of *B. cereus* extract, 2 ml of a solution containing 5 mg of adenosine (or 5 mg of adenosine + 5 mg of PRPP) in 0.2 M potassium phosphate buffer pH 7.0. At various time intervals 0.2 ml aliquots were withdrawn and added to 0.050 ml of 50% (w/v) trichloroacetic acid. The mixtures were centrifuged at room temperature and 0.050 ml of the supernatant fluid were chromatographed on Whatman no. 1 filter paper. With adenosine as substrate, inosine was found to be the first product followed by hypoxanthine. When PRPP was also present in the reaction mixture, formation of IMP was observed.

Adenosine deaminase and inosine phosphorylase activities were eluted from the G-100 column as symmetrical peaks. The elution volumes corresponding to inosine phosphorylase and adenosine deaminase from *B. cereus* yield values of 95,000 and 77,000 for their respective molecular weights assuming a globular structure. The first value is comparable with those obtained by Gardner and Kornberg [9] and by Engelbrecht and Sadoff [10]. On the contrary the molecular weight of *B. cereus* adenosine deaminase differs markedly from that of the enzyme from intestinal mucosa [12] (fig. 1).

In the experimental conditions employed for the determination of adenosine deaminase, no deamination could be detected when the following substrates were incubated with the peak material eluted from the G-100 column: adenine, AMP, 2'-AMP, 3'-AMP, ADP, ATP, GMP, 2'- and 3'-GMP mixed isomers, GDP, GTP, cytidine, CDP, CTP, ApA, ApG and GpA.

Both adenosine deaminase and inosine phosphorylase follow Michaelis–Menten kinetics, the K_m values being 7.1×10^{-5} M and 7.0×10^{-5} M, respectively.

Table 1 shows the results obtained on the inhibitory power of various nucleotides on inosine phos-

phorylase. Inhibitors may be devided into three main classes: 1) Purine nucleotides exerting 100% asymptotic inhibition: XMP, GTP, GMP, in the decreasing order (fig. 2A), the inhibition exerted by GMP being of the non-competitive type (fig. 3) with respect to inosine. When GMP and GTP were added together at equimolecular concentrations, the inhibition observed was greater than the sum of individual inhibitions (fig. 2A). 2) Purine nucleotides showing a finite asymptotic residual activity. Fig. 2B shows that varying concentrations of ATP or AMP result in sigmoidal inhibition curves with an asymptotic value around 50%. 3) Pyrimidine nucleotides, UTP, CTP, UMP and CMP also display an inhibitory effect on inosine phosphorylase, the nucleoside triphosphates being more effective. As can be seen in fig. 2C UTP and CTP show identical inhibition curves.

Hill coefficients higher than 2 have been calculated for inhibitors belonging to classes (1) and (2) (table 1).

None of the substances tested had any effect on *B. cereus* adenosine deaminase.

The chromatographic data show that the combined action of adenosine deaminase and inosine phosphorylase in *B. cereus* leads to the formation of hypoxanthine, which is in turn converted to inosinic acid, the common precursor of AMP and GMP, probably via an IMP pyrophosphorylase. The anabolic role of these enzymes is strengthened by the absence in *B. cereus* of xanthine oxidase and guanase which would convert hypoxanthine and guanine to degradation products; this leaves hypoxanthine and guanine available to participate in synthetic reactions leading to GMP and AMP. Furthermore, the inhibition exerted by these mononucleotides on inosine phosphorylase further suggests that the pathway may be operative in *B. cereus*.

Similar results were obtained in chick embryo brain [17].

The significance of the inhibition exerted by pyrimidine nucleotides remains obscure.

Acknowledgment

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