THE ROLE OF NUCLEOSIDE TRIPHOSPHATES IN THE CONTROL OF 3′- AND 5′-AMP DEPHOSPHORYLATION IN CRUDE EXTRACTS OF *B. SUBTILIS*

P. L. IPATA, C. A. PALMERINI*, S. SENESI, G. FALCONE and R. FELICIOLI**
Laboratorio di Biochimica, Facoltà di Scienze M.F.N. della Università, Via A. Volta 4, -56100 Pisa, Italy,
Istituto di Microbiologia dell'Università, Via S. Zeno 35, -56100 Pisa, Italy

Received 11 July 1975

1. Introduction

The regulation by nucleoside triphosphates of 5′- and 3′-ribomononucleotides dephosphorylation in mammalian cells and in microorganisms is well documented [1,7]. In spore forming *B. subtilis*, where both 3′- and 5′-mononucleotidases are present, and can be separated by gel filtration, the 3′-nucleoside phosphomonoesterase is about 1000 times less sensitive to nucleoside triphosphates inhibition than the 5′-nucleoside phosphomonoesterase [4]. The very low concentration of nucleoside triphosphate required to inhibit the latter enzyme (e.g. 0.1 μM ATP results in 50% inhibition) suggests that, at least in vegetative forms of *B. subtilis*, 3′- but not 5′-mononucleotides are dephosphorylated to free nucleosides.

In this paper we report a study on the dephosphorylation of 3′- and 5′-AMP added together to crude extracts of *B. subtilis* vegetative forms. The data clearly show that adenosine is formed from 3′- and 5′-AMP, in the absence of nucleoside triphosphates, but only from 3′-AMP, in the presence of μmolar nucleoside triphosphate concentration.

2. Materials and methods

Nucleotides, nucleosides and bases were obtained either from Sigma Chem. Co., St. Louis Mo., U.S.A., or from Boehringer und Soehne, Mannheim, Germany. Uniformely labeled [^{14}C] 5′-AMP was obtained from the Radiochemical Centre, Amersham, U.K. Adenosine deaminase and snake venom 5′-nucleotidase were purchased from Boehringer und Soehne and Sigma respectively. 3′-mononucleotidase, prepared from wheat germ, was a kind gift of Dr C. Borri Voltattorni. This enzyme preparation was insensitive to nucleoside-triphosphate inhibition, in the concentration range used in the course of the experiments reported here.

Preparation of crude extracts: cell-free extracts of vegetative forms of *B. subtilis* were prepared as previously described [4]. The protein content of extracts was determined according to Gornall et al. [8].

Assay conditions: the reaction mixture contained in a final vol. of 2100 ml, 140 μM 3′-AMP 140 μM 5′-AMP, 0.120 mM Tris-Cl buffer pH 7.2, and 2 mg of *B. subtilis* proteins. The effect of nucleoside triphosphates was investigated by including these compounds at the concentrations reported in the presentation of the experimental data. The temperature was 37°C. The molarities of substrates and inhibitors solutions were measured spectrophotometrically from their extinction coefficients at 260 nm at pH 7 [9].

Determination of 3′- and 5′-AMP: the time-dependent disappearance of 3′- and 5′-AMP was followed by a spectrophotometric procedure. 0.250 μl of the above described reaction mixture were withdrawn at different time intervals, and pipetted into a 1 cm path cuvet containing 30 μM CTP to inhibit the *B. subtilis* 5′-mononucleotidase [4], 2 μg of adenosine deaminase, and 0.2 mM Tris-Cl buffer, pH 7.8; the vol. was 0.8 ml.

The absorbance at 265 nm was recorded for several minutes; 10 μg of the 3′-mononucleotidase preparation

*From the Istituto di Chimica Biologica, Facoltà, di Farmacia, Università -06100 Perugia, Italy.

**From the Lab. per lo Studio delle Proprietà Fisiche di Biomolecole e Cellule, CNR, Via F. Buonarroti 9, -56100 Pisa, Italy.

were then added and the final change in absorbance at 265 nm was measured against a reference cuvet, in which 3′-AMP and 5′-AMP were omitted. 2 μg of snake venom 5′-mononucleotidase (which in separate experiments was found to be insensitive even to 1 mM nucleoside triphosphate) were then added, and the additional change in absorbance at 265 nm was again measured.

Chromatographic procedures: in some experiments, assays were carried out with 3′-AMP and [^{14}C] 5′-AMP. The reaction was halted with 10% perchloric acid, and the neutralized acid soluble material was applied to an AG 1x8 column (8.5 X 1.2 cm), formate form. Bases and/or nucleosides were eluted with about 80 ml H_2O; 3′- and 5′-AMP with about 30 ml of 1 M ammonium formate. The H_2O eluted material was concentrated under vacuum, and chromatographed on Whatman no 1 in *n*-butanol: ethanol: H_2O (4:1:5).

3. Results

Fig.1 shows the time dependent disappearance of 3′- and 5′-AMP added together to a crude extract of vegetative forms of *B. subtilis*. It can ce seen that the rate of 3′-AMP disappearance is about twice as fast as that of 5′-AMP. Addition of CTP at 23 μM did not affect the dephosphorylation of 3′-AMP, while completely abolished 5′-AMP breakdown. 2–10 μM UTP, 50 μM GTP, a mixture of 2 μM ATP, 0.2 μM CTP, 80 μM UTP and 50 μM GTP, as well as a mixture of the four nucleoside triphosphates, each at 12 μM, also abolished the production of adenosine from 5′- but not from 3′-AMP.

These results were confirmed by incubation a mixture of 3′-AMP and [^{14}C] 5′-AMP (specific radioactivity 1.5 X 10^6 cpm/μmole) with *B. subtilis* crude extracts, under the same incubation conditions described above. After 80 min at 37°C, the reaction products were analyzed by ion exchange chromatography (fig.2). When the incubations were carried out in the presence of nucleoside triphosphates, all added radioactivity was found associated with HCOOH eluted peak. This peak contained 5′-AMP but not 3′-AMP as shown by its sensitivity only to specific 5′-mononucleotidase. Furthermore its specific radioactivity was the same as that of the authentical [^{14}C] 5′-AMP used for the incubation mixture.

When incubations were carried out in the absence of nucleoside triphosphates, most of the radioactivity was associated to the H_2O eluted peak. Paper chromatography of this peak revealed the presence of adenosine (R_f = 0.28), identified after elution by its absorbance spectrum in acid and alkaline medium.

4. Discussion

3′-Ribomononucleotides are mainly formed by the action of 'spleen type' exonucleases (PDase II according to Razzel's terminology) [10] on RNA or of base

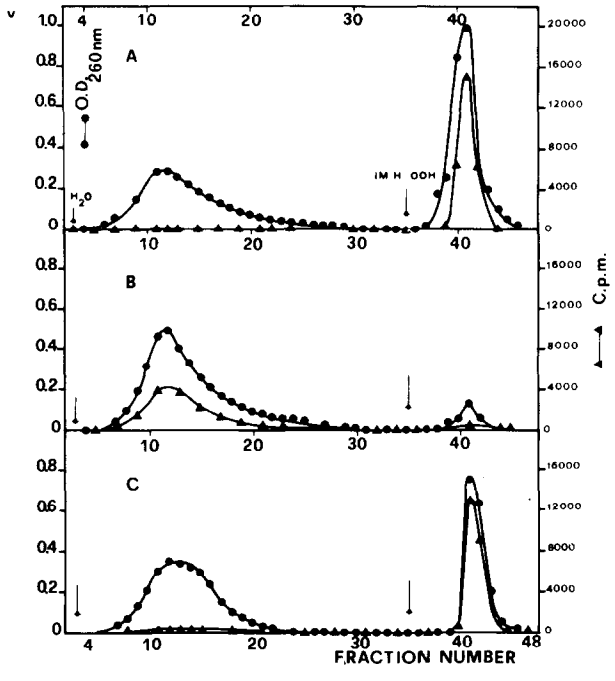

Fig.1. Time course of 3′-AMP and 5′-AMP disappearance in a crude extract of *B. subtilis*. The reaction mixture contained, in a final vol of 2 100 ml, 140 μM 3′-AMP and 140 μM 5′-AMP; 0.120 mM Tris-Cl buffer pH 7.2, and 2 mg proteins. At the times indicated in the abscissa, 0.250 ml portions of the reaction mixture were withdrawn, for the spectrophotometric determination of the residual 3′-AMP and 5′-AMP as described in the text under 'determination of 3′- and 5′-AMP'. Residual 3′-AMP (-●-●-) and 5′-AMP (-▲-▲-) in the absence of CTP. Residual 3′-AMP (-○-○-) and 5′-AMP (-△-△-) in the presence of 23 μM CTP.

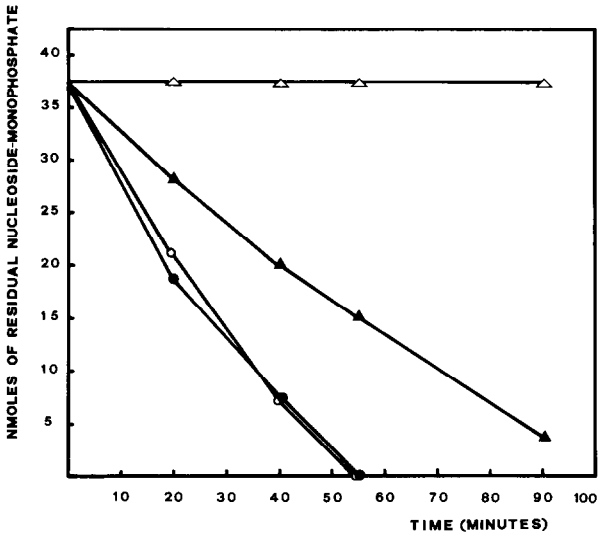

Fig. 2. Ion exchange separation of the acid soluble products formed when *B. subtilis* crude extracts are incubated with 3′-AMP and [^{14}C] 5′-AMP at equimolar concentrations. The reaction mixture contained, in a final volume of 2 100 ml, 140 μM 3′-AMP, 140 μM [^{14}C] 5′-AMP (1.5 × 10^6 cpm/μmole), 0.120 mM Tris-Cl buffer pH 7.2 and 2 mg of *B. subtilis* proteins. Mixtures were incubated 80 min. at 37°C. The reactions were halted with perchloric acid and the acid soluble products were analyzed as described in the text under 'chromatographic procedures'. In A and C the incubations were carried out in the presence of 23 μM CTP and of a mixture of ATP, UTP, GTP and CTP, each at 12 μM. In B the incubation was carried out in the absence of nucleoside triphosphates. Fractions of 4 ml were collected. Radioactivity was measured on 0.150 ml aliquots.

specific endonucleases acting on 5′-phosphodiester bonds of homologous sequences of RNA.

5′-Ribomononucleotides are formed (a) by the 'de novo' synthesis; (b) by the 'salvage' synthesis; and (c) by the action of 'snake venom type' phosphodiesterases (PDase I) [10] on RNA. Both 3′- and 5′-mononucleotides may be dephosphorylated by the action of specific nucleoside phosphomonoesterases.

Our results suggest that, at least in *B. subtilis*, the intracellular level of 5′-mononucleotides is regulated by nucleoside triphosphates, most likely through the strong inhibition exerted on 5′-mononucleotidase by these compounds [4]; 3′-mononucleotides are readily converted to nucleosides, and possibly to free bases, even at 'physiological' nucleoside triphosphates concentration. It must be pointed out in this respect that nucleosides and bases are converted to 5′-mononucleotides by the action of nucleoside kinases and nucleoside monophosphate pyrophosphorylases respectively, and that 5′-, but not 3′-mononucleotides are substrates of nucleoside monophosphokinases [11].

Therefore, nucleoside-triphosphates play a dual role in mononucleotide metabolism, acting as inhibitors of 5′-mononucleotides dephosphorylation, and as substrates of nucleoside and mononucleotide kinases.

As a consequence we propose that in *B. subtilis* endogenous free nucleosides and bases arise from 3′- but not from 5′-mononucleotides. This is suggested by the absence of radioactivity in bases and nucleosides when *B. subtilis* crude extracts are incubated with a mixture of 3′-AMP and [^{14}C] 5′-AMP, in the presence of μmolar concentration of nucleoside-triphosphates, and by the results presented in fig. 1, showing that even after prolonged incubation of *B. subtilis* crude extracts with both 3′- and 5′-AMP, in the presence of nucleoside triphosphates, only 3′-mononucleotides disappear.

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