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Characterization of the specificity of intracellular phosphodiesterases in *Bacillus subtilis*

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SUMMARY

Previously described methods for the spectrophotometric determination of phosphodiesterases (orthophosphoric diester phosphohydrolase, EC 3.1.4.1) [P.L. Ipata and R.A. Felicioli, *Eur. J. Biochem.*, 8 (1969) 174] have been employed to characterize intracellular phosphodiesterase in *Bacillus subtilis* during sporulation. Both 3′- and 5′-nucleoside monophosphate producing phosphodiesterases were found in vegetative and sporulating forms. In free spores the 3′-nucleoside monophosphate producing phosphodiesterase activity could not be detected with the methods employed.

It has been established that during the first 15 min after the addition of the germinating agent, the synthesis of nucleoside triphosphates in sporogenic bacteria is supported by enzymes for salvaging bases, nucleosides, and 5′-mononucleotides since the synthetic *de novo* pathways are not complete in the dormant spore[1—3]. These precursors could be made available in the germinating spores by the combined action of 3′-mononucleotide and 5′-mononucleotide producing phosphodiesterases (orthophosphoric diester phosphohydrolase, EC 3.1.4.1), nucleoside phosphomonoesterases and enzymes catalyzing base and nucleoside interconversion.

The previously described work on the spectrophotometric determination of 3′- and 5′-mononucleotide producing phosphodiesterases[4—7] led us to a systematic study of the levels of these enzymes during spore formation and germination.

Bacillus subtilis, strain ATCC 6633, was grown at 37 °C on a medium of the following composition: peptone, 5 g; beef extract, 3 g; yeast extract, 3 g; $MnSO_4$, 0.1 g; agar, 20 g; water to 1000 ml. Cells were harvested after 6, 14 and 24 h incubation, when

the vegetative forms represented 100%, 40%, and 0% of the bacterial population, and free spores were 0%, 2% and 100%, respectively; 58% of the cells at the 14th h of incubation were sporulating forms. Cells were washed 3 times with water by centrifugation at 2 °C, weighed, suspended in 2 vol. of 50 mM Tris–HCl buffer, pH 7, and disintegrated with glass beads in a Brown disintegrator continuously flushed with CO_2. The disrupted cells were centrifuged and a 20% solution of streptomycin sulfate (0.07 ml/10 mg of protein) was added to the clear supernatant fluid. After standing at 0 °C for 120 min the suspension was centrifuged at 25 000 x *g* for 30 min. The supernatant fluid was brought to 80% saturation with ammonium sulfate at pH 7.4, left for 12 h at 4 °C, and centrifuged. No nuclease activity was detectable in the supernatant fluid; the precipitate was dissolved in 50 mM Tris–HCl buffer, pH 7.4, and used as such for phosphodiesterase assays. The following dinucleoside monophosphates, containing adenosine or guanosine esterified either in 3′- or 5′-position and adjacent to a purine or pyrimidine base, were used as substrates: adenylyl-(3′→5′)-adenosine (ApA); adenylyl-(3′→5′)-guanosine (ApG); guanylyl-(3′→5′)-adenosine (GpA); cytidylyl-(3′→5′)-adenosine (CpA) and adenylyl-(3′→5′)-cytidine (ApC). The assay method is based on the spectrophotometric identification of adenosine or guanosine

TABLE I

3′- AND 5′-MONONUCLEOTIDE PRODUCING PHOSPHODIESTERASE IN *BACILLUS SUBTILIS*

The coupled optical enzyme assays were as follows: 0.6 ml of 50 mM Tris–HCl buffer, pH 7.4, 10 μl of dinucleoside monophosphate solution (1 mg/ml), and 100 μl of commercial adenosine deaminase (EC 3.5.4.4) (20 μg/ml) or 100 μl of the "guanosine revealing system"[6] were pipetted into one cuvette. The absorbance was recorded for 2 min and then the crude extract was added (150 μg protein); the mixture was rapidly mixed and the change in absorbance at 265 nm or at 290 nm was recorded against a reference cuvette, in which substrates were replaced by water. The final volume was 1 ml.

Substrate	*Possible products*	*Identified phosphodiesterase*	*Activity x 10^3* *		
			Vegetative forms	*Sporulating forms*	*Free spores*
CpA	Cp + A C + pA	3′-producing	65	150	0
GpA	Gp + A G + pA	3′-producing	50	120	0
ApA	Ap + A A + pA	3′-producing 5′-producing	110	250	22
ApG	Ap + G A + pG	5′-producing	45	450	10
ApC	Ap + C A + pC	5′-producing	70	200	20

*Activity is given as change in absorbance at 265 nm per min.

specifically released from either end of each dinucleoside monophosphate by the action of 3′ - or 5′-mononucleotide producing nucleases[6]. Spectrophotometric assays were carried out at 37 °C in 1-cm cuvettes, in a final volume of 1 ml, and the change in absorbance at 265 nm or at 290 nm was measured with a Perkin Elmer double beam recording spectrophotometer. Table I reports the specific activities of phosphodiesterases in the crude extracts of bacterial cells during the growth cycle. It can be seen that the phosphodiester bonds of dinucleoside monophosphates listed in Table I are cleaved to give 3′- and 5′-mononucleotides. In free spores the liberation of 3′-mononucleotides was not observed. This

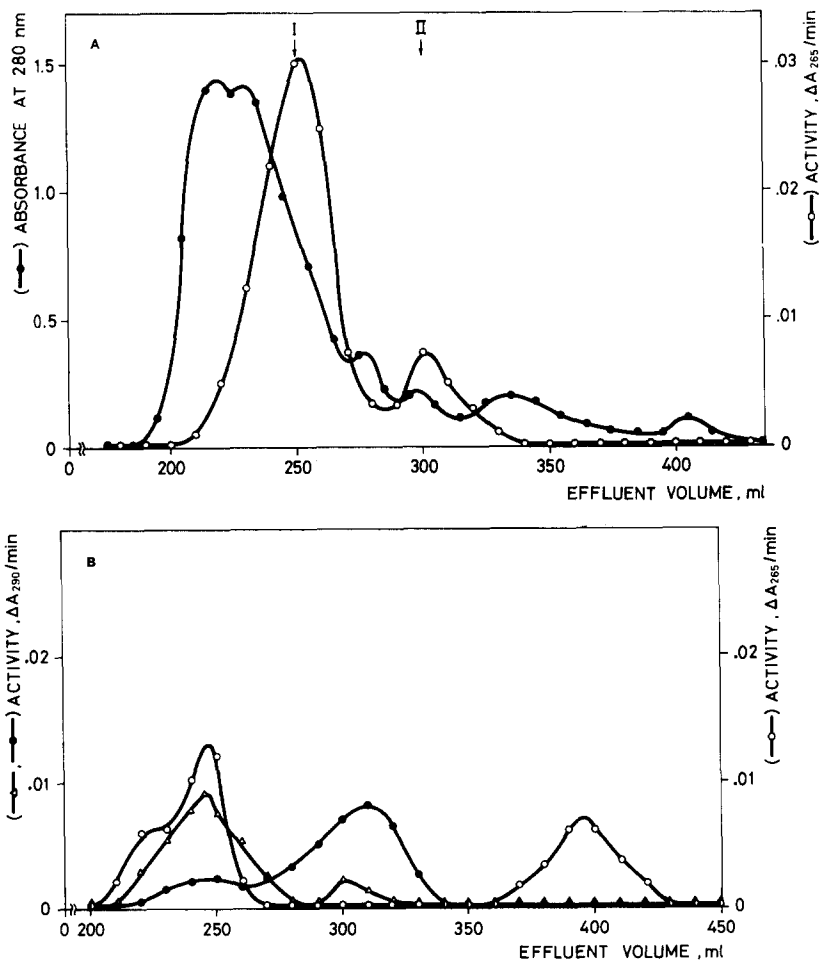

Fig. 1. 8 ml of the 80% ammonium sulfate fraction dissolved in 50 mM Tris–HCl buffer, pH 7.4, containing 15 mg protein/ml were eluted from a Sephadex G-100 column (2 cm x 90 cm) with the same buffer at a flow rate of 15 ml/h. Fractions of 5 ml were collected. Assays were carried out with 200 μl of each fraction as described in Table I. (A) ○—○, ApA as substrate, and adenosine deaminase as ancillary system; •—•, absorbance at 280 nm. (B) ○—○, CpA as substrate, and adenosine deaminase as ancillary system; △—△, ApG as substrate, and "guanosine revealing ancillary system"; •—•, GpA as substrate, and "guanosine revealing ancillary system".

might be ascribed to the presence of an inhibitor of the 3′-mononucleotide producing phosphodiesterase in the spore state. Furthermore, the presence of 3′-mononucleotide producing phosphodiesterases could not be excluded *a priori*, since theoretically 16 dinucleoside monophosphates should have been employed. However, (a) when Peak I and Peak II of Fig. 1A were assayed for phosphodiesterase activities in the presence of increasing amounts of crude spore extracts, no inhibition could be observed; (b) when yeast RNA was incubated with spore extracts, the acid-soluble reaction products were sensitive to 5′- (EC 3.1.3.5), but not to 3′-nucleotidase (EC 3.1.3.6), as shown by the following experiment. 5 mg of yeast RNA were incubated at 37 °C with 10 mg of spore proteins in 0.1 M Tris–HCl buffer, pH 7.2, in a final volume of 2 ml. At different time intervals, portions of 100 μl were withdrawn to follow the relase of acid-soluble (oligo)mononucleotides. After 40 min incubation, the reaction was stopped with 10% cold $HClO_4$ and the acid-soluble supernatant fluid neutralized with KOH. The precipitated $KClO_4$ was discarded and the supernatant fluid divided into two portions, which were incubated with excess commercial 3′- and 5′-nucleotidase, respectively, for 20 min at 37°C. Inorganic phosphate (1.38 μmoles) was released by the action of 5′-nucleotidase, but not by 3′-nucleotidase.

When the extracts obtained from cultures grown for 14 h were subjected to Sephadex G-100 column fractionation, two distinct enzyme activities catalyzing the hydrolysis of the phosphodiester bond of ApA could be detected (Fig. 1A). This suggests the presence of both 3′- and 5′-monophosphate producing phosphodiesterases:

$$\text{ApA} \xrightarrow{\text{3′-Monophosphate producing phosphodiesterase}} \text{3′-AMP + adenosine}$$

$$\text{ApA} \xrightarrow{\text{5′-Monophosphate producing phosphodiesterase}} \text{adenosine + 5′-AMP}$$

Each fraction was therefore assayed with ApG or GpA as substrates and "guanosine revealing ancillary system"[6]. The results reported in Fig. 1B suggest that Peaks I and II of Fig. 1A possess 3′-mononucleotide and 5′-mononucleotide producing phosphodiesterase activity, respectively.

Our results show that in vegetative and sporulating forms of *Bacillus subtilis* the RNA phosphodiester bond can be hydrolyzed to give both 3′- and 5′-nucleoside monophosphate, while in the spore RNA can be hydrolyzed to give only 5′-mononucleotides, as precursors of the nucleoside triphosphates in the early stages of germination.

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