INTRACELLULAR RIBONUCLEASE OF BACILLUS SUBTILIS; SPECIFIC INHIBITION BY ATP AND dATP

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Summary

Ribonuclease activity in the crude extract of Bacillus subtilis was strongly inhibited by low concentration of ATP.

Ribonuclease in the extract was purified by 2100 times to almost homogeneous state as tested by disc electrophoresis.

The purified enzyme hydrolyzed RNA to Ado-2':3'-P, Guo-2':3'-P, Cyd-2':3'-P, Urd-2':3'-P and was specifically inhibited by ATP and dATP. K_i values for ATP and dATP were 0.12 and 0.21 μM respectively. The mode of inhibition of ATP was competitive.

Introduction

In the course of studying a cell free protein synthesizing system of B. subtilis K with special reference to ribonuclease synthesis, we found that ribonuclease activity in the reaction mixture could not be detected in the presence of ATP and ATP generating system, but was observable in their absence. It became clear that ribonuclease activity in the enzyme preparation (dialyzed S-100 fraction (1)) was strongly inhibited by the addition of ATP (2). In an effort to elucidate the nature of ribonuclease in the crude extract, the enzyme was purified by 2100 times to almost homogeneous state as tested by disc electrophoresis.

The present communication deals with the purification of intracellular ribonuclease of B. subtilis and the specific

inhibition of the enzyme by ATP and dATP. Such an ATP-controlled ribonuclease, as far as we know, has not been isolated from microorganisms, although Suskind et al (3) briefly reported the inhibitory effect of ATP on the intracellular ribonuclease activity of Neurospora crassa.

Methods

Purification of intracellular ribonuclease. B. subtilis K was grown in nutrient broth for 17 hours at 30°C. About 500 g (wet weight) of washed cells were disrupted by a French pressure cell at 100 atm/cm^2. After centrifugation at 75,000 x g for 90 min, the supernatant was applied to DEAE-Sephadex A-50, Amberlite IRC-50, and Sephadex G-75 columns. As shown in Table I, the intracellular ribonuclease was purified by 2100 times and recovery was 26%. The details of the purification procedure will be published elsewhere.

Enzyme assay. Ribonuclease activity was assayed according to Anfinsen et al (4). Standard reaction mixture contained 3 mg of yeast RNA (Sigma Chemical Co.), 40 μ moles of phosphate buffer pH 5.7 and an appropriate amount of enzyme in one ml of total volume. Incubated for 30 min at 30°C, the reaction mixture was added with 0.3 ml of uranyl acetate reagent (0.75 % uranyl acetate in 25% perchloric acid) and stood for 20 min at 5°C. After centrifugation at 3000 rev/min for 20 min, 0.2 ml of the supernatant obtained was diluted to 5.0 ml and absorbancy at 260 mμ was measured. One unit of ribonuclease was defined as the amount which increased optical density at 260 mμ by 1.0 at the standard assay condition. Assay was carried out in duplicate.

Deoxyribonuclease activity was measured similarly using native or heat-denatured calf thymus DNA (Sigma Chemical Co.,

Table I Purification procedure of the intracellular ribonuclease of B. subtilis.

Step	O.D. (at 280 mμ)	Volume (ml)	Unit/ml	Specific activity (unit/O.D.)	Total unit
Crude extract	80.0	460	11.05	0.138 (1)	5000
1st DEAE	13.6	60	56.0	4.11 (29)	3350
2nd DEAE	0.58	590	4.15	7.20 (52)	2450
3rd DEAE	1.80	15.4	113.5	65.5 (474)	1850
Amberlite IRC-50	0.056	155	9.15	162 (1170)	1410
Sephadex G-75	0.110	40	32.0	290 (2100)	1305

Ribonuclease activity was assayed in a reaction mixture containing per ml 3 mg yeast RNA and 40 μ moles of phosphate buffer pH 5.7 at 30°C. Reacted for 30 min, the reaction mixture (one ml) was added with 0.3 ml of uranyl reagent (0.75% uranyl acetate in 25% perchloric acid). The absorbancy at 260 mμ of 1/25 diluted acid soluble fraction was measured.

One unit of ribonuclease was defined as the amount which increased the optical density at 260 mμ by 1.0 at the assay condition mentioned above.

final 0.1%) as the substrate. Phosphodiesterase activity was measured by determining the absorbancy of liberated p-nitrophenol at 420 mμ employing bis-p-nitrophenyl phosphate (0.5 mM in 0.1 M acetate buffer pH 5.7) as the substrate. Phosphatase assay was done similarly except that p-nitrophenyl phosphate (final 8 mM) was the substrate.

Disc electrophoresis. Disc electrophoresis was carried out at 2 mA/tube for two hours at 20°C using 7.5 gel (5).

Thin layer chromatography. Thin layer chromatograms were run using cellulose powder thin layers (2.5 x 7.5 cm) and n-propanol: conc. NH_4OH: water (70:10:25 v/v/v) as the solvent.

R_f values for Ado-2':3'-P, Cyd-2':3'-P, Urd-2':3'-P and Guo-2':3'-P were 0.63, 0.51, 0.44 and 0.36 respectively.

Results

Several properties of intracellular ribonuclease. The purified intracellular ribonuclease of B. subtilis K was

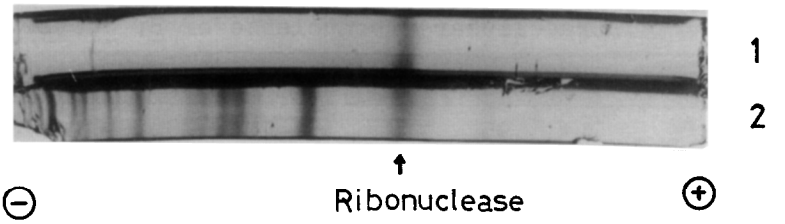

Fig. 1 Disc electrophoretic patterns. About 60 μg protein of the purified ribonuclease (1) and 180 μg protein of the crude extract of B. subtilis K (2) were migrated at 2 mA/tube for 2 hours in 7.5 gel. Protein bands were stained with Amidoschwartz 10B.

almost homogeneous as tested by disc electrophoresis (Fig. 1).

The enzyme had no deoxyribonuclease, phosphodiesterase, and phosphatase activities. The optimum pH was at 5.5-5.7 as reported by Nishimura et al (6). The ribonuclease hydrolyzed yeast RNA by 95% at the standard assay condition and at 2.5 unit of the enzyme per ml within two hours. The reaction products were Ado-2':3'-P, Guo-2':3'-P, Cyd-2':3'-P and Urd-2':3'-P as tested by thin layer chromatography. Even after prolonged incubation these 2',3'-cyclic nucleoside monophosphates were not converted to corresponding 2'- or 3'-nucleotides.

Effect of nucleotides. As shown in Table II, the intracellular ribonuclease was strongly and specifically inhibited by ATP and dATP, i.e., 0.1 mM of either nucleotide inhibited the enzyme by 95% at pH 5.7. ADP was a less effective inhibitor. The inhibition by these nucleotides was more pronounced at pH 7.5 than at pH 5.7, e.g., 2 μM of ATP inhibited the ribonuclease by 41% and 80% at pH 5.7 and 7.5, respectively. Fig. 2 shows the time course of RNA degradation by the enzyme in the presence or absence of 10 μM ATP or dATP.

Table II The effect of nucleotides on the intracellular ribonuclease of B. subtilis.

Nucleotide	% Inhibition	Nucleotide	% Inhibition
ATP	95	5'-AMP	0
GTP	9	5'-GMP	0
CTP	0	5'-CMP	0
UTP	3	5'-UMP	0
ITP	4	5'-IMP	0
dATP	94	3',5'-AMP	0
ADP	55	3'(2')-AMP*	0
GDP	20	3'(2')-GMP*	0
CDP	2	3'(2')-CMP*	0
UDP	0	3'(2')-UMP*	0

The concentration of nucleotides was 0.1 mM. Assay conditions were as given in Table I and 0.25 unit of ribonuclease was added to one ml of the reaction mixture. * Mixture of 3'- and 2'-nucleotides.

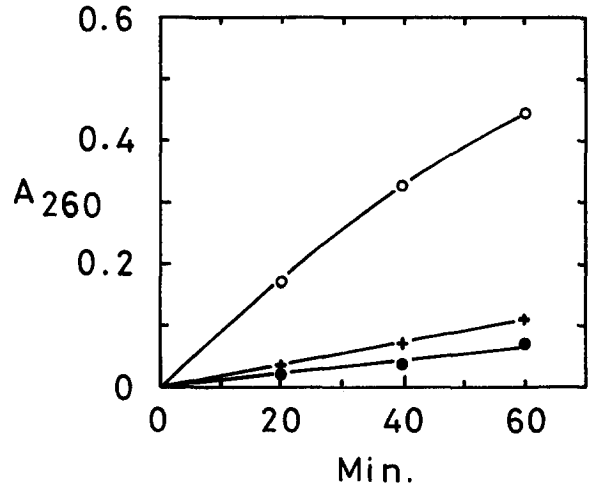

Fig. 2 Time course of RNA degradation by ribonuclease in the absence or presence of inhibitory nucleotides. Assay conditions were as given in Table I except that reaction period was varied. —o— ; control —•— ; plus 10 μM ATP (Sigma), —+— ; plus 10 μM dATP (Sigma).

As shown in Fig. 3 (A and B), the modes of inhibition of ATP and ADP were competitive, and that of dATP was nearly competitive. K_i values for ATP and dATP were 0.12 and 0.21 μM respectively, and that of ADP was 5.2 μM.

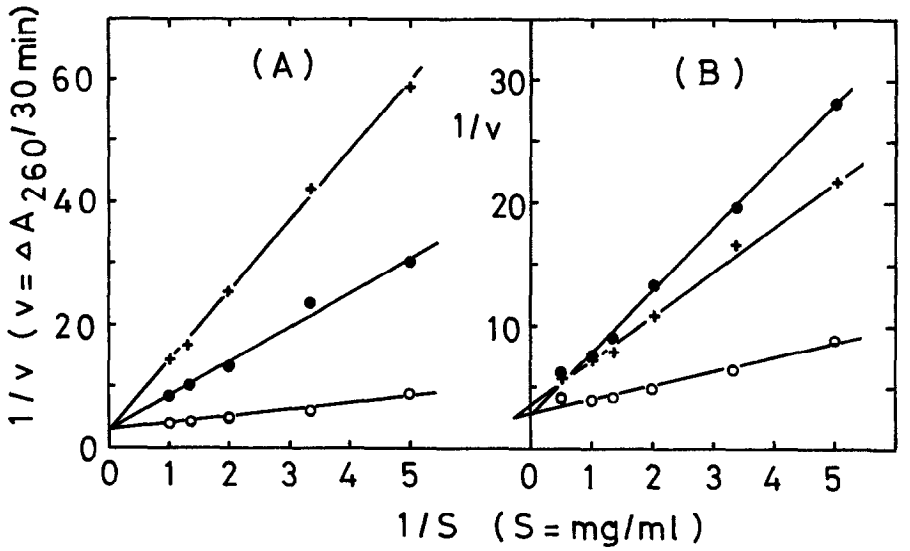

Fig. 3 Double-reciprocal plots of RNA concentration and initial reaction velocities in the absence or presence of nucleotides. Assay conditions were as given in Table I and 0.25 unit of ribonuclease was added to one ml of the reaction mixture.
(A): —o— ; control, —•— ; plus 0.5 μM ATP, —+— ; plus 50 μM ADP. (B): —o— ; control —•— ; plus 0.5 μM ATP, —+— ; plus 0.5 μM dATP.

Discussion

The intracellular ribonuclease of B. subtilis was previously purified by 17 times by Nishimura et al (6). But the inhibitory effect of ATP or dATP had not been reported. As far as examined, ribonuclease activity in the crude extract of other five strains of B. subtilis were all inhibited by ATP (7). The inhibitory effect of ATP on the intracellular ribonuclease, therefore, is thought to be common to B. subtilis species.

It may safely be said that intracellular concentration of ATP in bacteria is above 0.1 mM judging from the data of several investigators (8,9,10). Most of intracellular ribonuclease, therefore, is thought to be inhibited in B. subtilis

cells, although actual pool size of ATP was not yet measured.

ATP probably acts as natural regulator or inhibitor of the intracellular ribonuclease in B. subtilis.

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