PROTEOLYTIC CONVERSION *IN VITRO* OF *B. SUBTILIS* VEGETATIVE RNA POLYMERASE INTO THE HOMOLOGOUS SPORE ENZYME

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1. Introduction

It was previously shown that the structural difference between the two DNA-dependent RNA polymerases isolated from *B. subtilis* vegetative cells and from dormant spores resides in the replacement of one of the β subunits in the vegetative enzyme (146,000 daltons each) by a polypeptide with the molecular weight of 129,000 daltons present in the spore RNA polymerase [1]. A similar difference in the RNA polymerase was observed by Losick and Sonensheim in sporulating cells of *B. subtilis* strain 3610 [2].

One of the questions raised by these findings was how the β modification could take place *in vivo*. By analogy to the studies of Sadoff et al. [3] on a protease catalyzed interconversion of the *B. cereus* vegetative aldolase into the spore form of the same enzyme, it was suggested that a similar mechanism may operate during sporulation leading to the observed RNA polymerase modification. This hypothesis was further strengthened by the experiments reported by Leighton et al. in which they observed the β modification *in vitro* by incubating purified *B. subtilis* vegetative RNA polymerase in the presence of the "serine protease" isolated from the same microorganism [4]. However, the question which was still left to be clarified was to know how the serine protease which, as shown by Millet [5] is an extracellular enzyme, could act *in vivo* on the DNA-dependent RNA polymerase located in the nuclear fraction of the cell [6]. Therefore, we thought that, *in vivo*, the proteolytic interconversion of the vegetative RNA polymerase into the spore form, should rather be catalyzed by an intracellular protease which, as shown recently by Millet, is present in *B. megaterium* and *B. subtilis* [7].

In the present communication we report the results obtained by incubating the purified RNA polymerase from *B. subtilis* vegetative cells in the presence of pure intracellular protease isolated from *B. megaterium*. These results clearly show that the structural modification observed in the spores in one of the β subunits can be obtained *in vitro*. We also show that the *in vitro* modified RNA polymerase retains the capacity to transcribe different DNA templates as does the spore enzyme. We finally show that the extracellular serine protease from *B. subtilis*, when present in the incubation mixture at a concentration about 100 times lower than the intracellular enzyme, hydrolyzes the RNA polymerase to small molecular weight material.

2. Materials and methods

The vegetative RNA polymerase was purified from exponentially growing cells of *B. subtilis* Marburg, 12A (isolated by Professor J. Spizizen) by the method of Burgess [8]. In some experiments the DEAE-cellulose step was followed by a glycerol gradient (10–30%) centrifugation. Only the peak fractions with the highest specific activity were pooled and stored in 50% glycerol at −20°.

In the particular preparation used in the present experiments the content of the sigma subunit was lower than usual.

The RNA polymerase from cleaned dormant spores of *B. subtilis* was prepared as previously described [1, 9].

Before use in the protease incubation mixture, an aliquot (usually 0.3 ml) of the RNA polymerase was dialyzed 140 min against 1 l of buffer containing: 10 mM Tris-HCl pH 7.9; 10 mM $MgCl_2$; 5 mM 2-mercaptoethanol; 0.1 ml Na-EDTA; 1 mM $CaCl_2$ and 5% glycerol (buffer I). The assay mixture for RNA polymerase contained in 0.5 ml: 40 mM Tris-HCl buffer pH 7.8; 2 mM $MgCl_2$; 4 mM 2-mercaptoethanol; 0.4 mM K_3PO_4; 50 mM KCl; GTP, CTP, UTP and ^{3}H-ATP (7,700 cpm/nmole), 0.25 mM of each.

The intracellular protease was purified from *B. megaterium* strain MA-31 (Ade^-Meg^-) essentially by the procedure already described [7]. An additional step was included which yielded a pure preparation as judged by the single protein band containing the proteolytic activity obtained on acrylamide gel electrophoresis [10]. This procedure was finally based on the following steps: a) precipitation of nucleic acids by streptomycin; b) ammonium sulfate fractionation; and c) chromatography on DEAE-cellulose. Further purification was carried out on a small scale by polyacrylamide gel electrophoresis. An aliquot of about 15 μg protein from step c) containing 60 "azocoll" units (an "azocoll" unit is defined as the amount of protein catalysing the hydrolysis of 1 μg of "azocoll" per min) was layered on each polyacrylamide gel (7%) and subjected to electrophoresis (2 mA/gel at 4°) for 7 hr. The single protein band containing the activity was separated from the rest of the gel, kept at −20° and utilized when required. Immediately before use, the gel fraction was homogenized in 0.2 ml Tris-HCl–Ca buffer (Tris-HCl, pH 7.3 and 2 mM $CaCl_2$). The supernatant, containing pure protease freed from the gel granules was used in the different assays. Usually 35–40% of the original activity layered on the gel was recovered in the supernatant.

3. Results and discussion

3.1. *Treatment of vegetative RNA polymerase by intracellular protease from B. megaterium*

The choice of the *B. megaterium* mutant MA-31 for the isolation of the intracellular protease was of particular importance for the experiments described here, for this mutant is defective in the single extracellular protease termed megateriopeptidase [7], present in the wild strain. As a result, contamination of the purified enzyme with extracellular protease activity was completely avoided. This was not the case when *B. subtilis* was used for isolation of the intracellular protease. In the case of *B. subtilis*, the same purification procedure as for *B. megaterium* yielded an enzyme much less stable and still contaminated with traces of extracellular proteolytic activity. When *B. subtilis* intracellular protease was incubated with RNA polymerase, we observed, in addition to the β modification, some degradation of all the subunit components of the enzyme.

Therefore, in the experiments reported in this communication, the intracellular protease of *B. megaterium* was used under the following conditions: about 5 units of protease were incubated with 15 μg of RNA polymerase in a volume of 130 μl containing buffer I for 30 min at 37°. The reaction was stopped by immersing the incubation mixture in ice followed by the addition of 5 μl of a solution of *p*-toluene sulphonyl fluoride (6 mg/ml) and 15 μl of concentrated glycerol to inhibit completely the proteolytic activity. An aliquot was then taken out for analysis by acrylamide gel electrophoresis of the structural modification of the RNA polymerase. The transcriptional activity of the remaining modified RNA polymerase was tested by adding the components of normal RNA polymerase assay as described in "Material and methods".

When pure vegetative RNA polymerase was incubated with *B. subtilis* extracellular serine protease, even at a concentration of 0.05 "azocoll units" for 2 min at 0°, almost complete degradation of the polymerase was observed (fig. 1C).

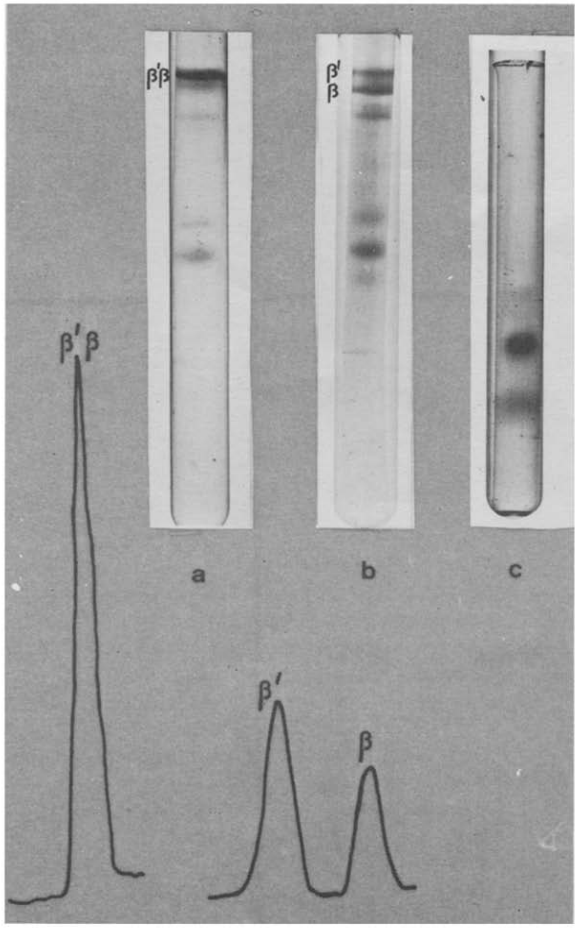

Fig. 1. β subunits of *B. subtilis* vegetative RNA polymerase: a): control; b): after treatment with intracellular protease of *B. megaterium*; c): after treatment with extracellular protease of *B. subtilis*. Electrophoresis in 0.1% SDS polyacrylamide gels (5%). After destaining only the β protein bands were scanned in Gilford scanner at 515 nm.

Similar results were obtained when the serine protease was replaced by trypsin (TPCK, Worthington Biochem. Corp.) at the concentrations of 5 or 0.02 μg per test containing 25 μg of polymerase.

Fig. 1 shows the subunit structure of the vegetative *B. subtilis* RNA polymerase before (a) and after (b) treatment with the intracellular protease. It can be seen that one of the β polypeptide bands present in the control (146,000 daltons each [1]) is replaced by a protein band with a lower molecular weight (120,000 daltons). From the electrophoregram shown on the same figure we found that the molar ratio of the α subunit (43,000 daltons) to the β′ (146,000 daltons) and to the β modified subunit (120,000 daltons) was 1:0.5:0.5, similar to that observed for the *B. subtilis* spore RNA polymerase [1].

Table 1 shows the transcriptional activity of the *in vitro* modified RNA polymerase compared to that of the untreated enzyme, on one hand, and to the spore enzyme, on the other hand. It can be seen that the β modification either made *in vivo* (spore enzyme) or obtained *in vitro* by the proteolytic interconversion of the vegetative enzyme does not significantly change the ratio of activities of T_4, *B. subtilis* and Φe DNA's to the calf thymus DNA used as templates. It is interesting however, to notice that the transcription of d-AT by the spore enzyme, and to a lesser extent, by the modified enzyme, is considerably increased as compared to the vegetative RNA polymerase. This suggests that the modification of the β subunit, in some way, affects the transcription of a specific segment of the DNA.

In some experiments, when holoenzyme was used in the protease–RNA polymerase incubation mixture, we observed that in addition to the β modification, some degradation of the sigma protein factor takes place. The α subunits were apparently never affected by the protease treatment. It is quite possible that inside the cell a protective mechanism might exist which prevents the RNA polymerase against further proteolytic damage. The studies by Novak and Doty have shown that, during transcription, the resistance of the RNA polymerase from *Micrococcus lysodeikticus* to proteolytic enzymes increased sharply when the enzyme is in the binary complex DNA–RNA polymerase or in the ternary complex DNA–RNA polymerase–RNA [11]. In similar experiments, Lecocq has shown that the loss of DNA binding capacity and of polymerase activity resulting from the treatment of the enzyme by trypsin, can be prevented by preincubation with DNA [12].

From data reported here it seems clear that the *in vitro* modified RNA polymerase mimics both the size of the β polypeptide and the transcriptional activity of the spore enzyme. These results therefore provide strong evidence that the mechanism of proteolytic interconversion might well be the one by which the vegetative RNA polymerase is modified *in vivo* during sporulation.

Table 1
Activity of vegetative RNA polymerase treated and non-treated by *B. megaterium* intracellular protease.

	Template DNA					$\frac{T_4}{\text{thymus}}$	$\frac{B.\ subtilis}{\text{thymus}}$	$\frac{\phi e}{\text{thymus}}$	$\frac{\text{d-AT}}{\text{thymus}}$
	Calf thymus	T_4	*B. subtilis*	ϕe	d-AT				
Vegetative RNA* polymerase	112	68	220	467	254	0.6	1.96	4.1	2.2
Spore polymerase	180	170	140	–	2 100	0.94	0.77	–	11.60
Modified vegetative polymerase	53	100	97	180	185	1.05	1.0	1.9	3.6

Assay conditions as described in "Material and methods". 40 μg A_{260} units was used per test. Activity is expressed in units/mg of protein.

* This preparation contained lower amounts of sigma factor than usual.

The exact significance of the modification for the sporulation process is a question which still remains to be answered.

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