Initiation of Bacterial Spore Germination

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To investigate the problem of initiation in bacterial spore germination, we isolated, from extracts of dormant spores of Bacillus cereus strain T and B. licheniformis, a protein that initiated spore germination when added to a suspension of heat-activated spores. The optimal conditions for initiatory activity of this protein (the initiator) were 30°C in 0.01 to 0.04 M NaCl and 0.01 M tris(hydroxymethyl)aminomethane (pH 8.5). The initiator was inhibited by phosphate but required two cofactors, L-alanine (3/4 of K_m for L-alanine-initiated germination) and nicotinamide adenine dinucleotide (1.25 X 10^{-4} M). In the crude extract, the initiator activity was increased 3.5-fold by heating the extract at 65°C for 10 min, but the partially purified initiator preparation was completely heat-sensitive (65°C for 5 min). Heat stability could be conferred on the purified initiator by adding 10^{-3} M dipicolinic acid. A fractionation of this protein that excluded L-alanine dehydrogenase and adenosine deaminase from the initiator activity was developed. The molecular weight of the initiator was estimated as 7 X 10^6. The kinetics of germination in the presence of initiator were examined at various concentrations of L-alanine and nicotinamide adenine dinucleotide.

L-Alanine-initiated germination of activated bacterial spores is an example of a biological trigger mechanism. A statistical analysis of the initial events in spore germination has recently led to the proposal that spores contain an allosteric enzyme that controls the rate-limiting step in germination (9, 22).

1 L-Alanine dehydrogenase (8), alanine racemase (2), proteolytic enzymes (18), and muramidase-like enzymes (5, 19) have all been proposed as possible candidates for the initiating enzyme. Since all the properties of germination cannot be explained by any one of these enzymes, a search was undertaken for a heat-activated initiator in spores of Bacillus cereus T. This paper describes the partial purification and some of the properties of an initiator protein. A preliminary report of this study has been presented (J. C. Vary, Bacteriol. Proc., p. 37, 1965).

Materials and Methods

Organism. B. cereus T spores were grown in a modified G medium (CaCl_2, 100 mg/liter) at 30°C for 36 hr (2). The spores were separated from the debris by centrifugation, washed 15 to 20 times, and lyophilized.

Heat activation and germination. Spores were suspended in distilled water to an optical density (OD) of 1.5 to 2.0 at 625 μM and were heat-activated (5 hr at 65°C or 20 min at 75°C); the spores were washed twice with distilled water and resuspended in 0.05 M tris(hydroxymethyl)aminomethane (Tris) buffer (pH 8.5). During the course of the assays, the stock of heat-activated spores was stored at 0°C, and samples were removed and equilibrated at 30°C for 10 min before use.

Germination was measured by continuously following the decrease in OD at 625 μM (21). The suspension of spores was added to a cuvette which contained buffer and initiating reagents so that the initial OD was 0.8 to 0.9. Several methods for calculating characteristic parameters from a germination curve have been devised (14, 21). One method, not previously described, is a calculation of the time required for the OD to decrease by 10% of its initial (OD_0) value. The OD_{100} equals 0.90 OD. The OD_{100} equals 0.90 OD. The OD_{100} calculation is arbitrary, but it has the advantage of convenience and proportionality to both lag time and rate of OD decrease. In addition, the value OD_{100} is a reciprocal velocity (minutes/10%) parameter and may, therefore, be used directly in reciprocal plots. The characteristics and limitations of the OD_{100} calculations have been discussed elsewhere (J. C. Vary, Ph.D. Thesis, Univ. of Wisconsin, Madison, 1967). A unit of initiator activity was defined as that amount of extract which caused a 10% reduction in the initial OD in 10 min.

Preparation of extracts. Spore extracts were prepared by grinding an aqueous suspension of spores (200 mg/ml) with Superite glass beads, 120 μm in diameter (Minnesota Mining & Manufacturing Co., St. Paul, Minn.) at a ratio of 1:1 (v/v) in a Mini-Mill.

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(Gilford-Wood Co., Hudson, N.Y.). The suspension was ground at top speed for 8 min at 0 C with a gap setting between the rotor and stator of 0.025 inches (0.06 cm). Usually, 200 to 300 ml of a spore suspension was broken in 50-ml samples.

The extract and debris were decanted from the glass beads and were centrifuged at 12,000 × g for 10 min at 0 C. The supernatant fluid was centrifuged again at 12,000 × g, and this second supernatant solution is referred to as the crude extract. Attempts to remove nucleic acids from the crude extract by either streptomycin sulfate or MnCl₂ precipitation led to inactive preparations. Thus, the nucleic acids were digested with a mixture of four nucleases: pancreatic deoxyribonuclease, phosphodiesterase, T₁ ribonuclease, and pancreatic ribonuclease at final concentrations of 25, 25, 450, and 450 µg/ml, respectively. After 45 min at 25 C, the incubation mixture was adjusted to 0.01 M with Tris (pH 8.5) and was precipitated with (NH₄)₂SO₄. Solid (NH₄)₂SO₄ was slowly added with mixing at 25 C until 100 % saturation was reached. After 30 min of stirring, the mixture was centrifuged at 10,000 × g for 10 min at 25 C. The precipitate was suspended in 0.1 M NaCl-0.01 M Tris (pH 8.5), and about 10 ml was dialyzed for 24 hr in 1.0 liter of 0.1 M NaCl-0.01 M Tris (pH 8.5) at 0 C with three changes of buffer.

Assays. Protein was determined either colorimetrically (12) with bovine serum albumin as a standard or by the ratio of absorbancies at 280 and 260 mµ on a Beckman DB spectrophotometer. Dipicolinic acid (DPA) was assayed by the method of Janssen, Lund, and Anderson (10); L-alanine dehydrogenase, by the method of McCormick and Halvorson (15); adenosine deaminase, by the method of Kakekar (11); and yeast alcohol dehydrogenase, by the method of Vallee and Hoch (20).

Materials. The following enzymes were used: lysozyme, 20,000 units/mg, Grade I (Sigma Chemical Co., St. Louis, Mo.); Pronase, 45,000 units/mg (Sigma Chemical Co.); bovine pancreatic ribonuclease, Grade A (Calbiochem, Los Angeles, Calif.); T₁ ribonuclease, Grade B (Calbiochem); phosphodiesterase, Type II (Sigma Chemical Co.).

The following reagents were used: DPA (Aldrich Chemical Co., Inc., Milwaukee, Wis.), ninyhdrin (Sigma Chemical Co.), (NH₄)₂SO₄ (Special Enzyme Grade, Mann Research Laboratories, New York, N.Y.), Tris (Sigma Chemical Co.), nicotinamide adenine dinucleotide (NAD) and NAD analogues (P-P Biochemicals, Inc.), bovine serum albumin (Fraction V, Calbiochem), horse heart cytochrome c (Sigma Chemical Co.), Azo-albumin (Nutritional Biochemicals Corp., Cleveland, Ohio), Blue Dextran 2000 and Sephadex 200 (Pharmacia Inc., New Market, N.J.).

RESULTS

Isolation of initiator activity. In the initial stages of this investigation, we found that the initiator had a negligible effect on dormant spores, whereas the germination of heat-activated spores was susceptible to initiator activity. The reason for the inactivity of initiator on dormant spores is not understood, but it is assumed that the initiator cannot enter the spore unless the spore is activated.

In a search for a convenient source of initiator activity, we found that extracts from dormant spores contained a heat-activated substance which initiated the germination of activated spores. Extracts from heat-activated spores yielded a partially activated initiator, whereas the exudate of germinated spores did not.

A crude extract was prepared from dormant spores and assayed, as described in Materials and Methods. The following properties were determined. (i) The optimal temperature for initiator activity was 30 C, the optimal time of heat activation at 65 C was 10 min, and the optimal pH was 8.5 (Table 1). (ii) Both the crude extract and the heat-activated extract lost activity when they were stored at 0 C for 4 days. (iii) Heat-activated extracts, dialyzed overnight at 0 C, were completely devoid of activity. However, the initiator activity could be restored to the dialyzed extract by the addition of boiled extract of dialyase. Apparently dialysis removed cofactors that were required for initiator activity. We found that, if a dialyzed extract was assayed in the presence of L-alanine, 48 % of the initiator activity in the crude extract was recovered. We also observed that NAD had a stimulatory effect on initiator activity. When a dialyzed extract from dormant spores was used, the NAD content of the extract was found to be 100 %.

![Table 1](http://jb.asm.org/Downloaded from http://jb.asm.org/)
extract was assayed with L-alanine and NAD, almost 100% of the original activity was recovered (Fig. 1). The concentration of L-alanine (10^{-3} M) used in these experiments did not, by itself or in the presence of NAD, initiate germination. This concentration of L-alanine was sevenfold less than the \( K_a \) for L-alanine-initiated germination. Also, NAD and the dialyzed crude extract did not initiate germination.

Isolation of initiator from B. licheniformis. To test the possibility that the initiator substance might be species specific, the method of extraction of initiator developed for B. cereus (described below) was applied to spores of B. licheniformis (a gift of Ian MacKechnie). We assayed the fractionated extract for initiator activity (Fig. 2) and found that extracts from dormant spores of B. licheniformis initiated the germination of B. cereus spores. Thus, B. cereus T spores do not possess a unique initiator substance.

Separation of lytic and initiator activities. Since a lytic enzyme from B. cereus was shown to initiate nonphysiological germination in B. cereus (5), the possibility was investigated that this initiator might be the same or a similar enzyme. A crude extract was fractionated by the procedure of Gould and Hitchins (5). When the various fractions in this procedure were assayed for initiation of germination (heat-activated spores in buffer), activity was apparent only in the material which was soluble in 60% saturated (NH_4)_2SO_4. This activity was heat-labile and was completely inhibited by 0.1 M phosphate. When these same fractions were assayed for nonphysiological germination (5), activity was observed in the pH 3.5 soluble fraction only. These results are summarized in Table 2. Since initiator activity for physiological germination could be distinguished from enzyme activity for nonphysiological germination, the initiator is probably not the Strange and Dark S-enzyme isolated by Gould and Hitchins (5).

Fractionation of the initiator. A crude extract was prepared and treated with nucleases followed by (NH_4)_2SO_4 precipitation, as described in Materials and Methods. Initiator extracted by these methods was heat-sensitive (65 C for 5 min). The dialyzed initiator preparation was
TABLE 2. Fractionation of the crude extract for the Strange and Dark enzyme and the initiator

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Germination activitya</th>
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<tbody>
<tr>
<td>(NH₄)₂SO₄ 60% (soluble)</td>
<td>14.5 3.9</td>
</tr>
<tr>
<td>pH 3.5 (soluble)</td>
<td>1.0 46.2</td>
</tr>
<tr>
<td>pH 3.5 (insoluble)</td>
<td>1.5 3.9</td>
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- Measured as decrease (%) in optical density in 30 min for 0.5 ml of each fraction.
- Spores of Bacillus cereus T were broken, and the extract was fractionated by the method of Gould and Hitchins (5). The 60% saturated (NH₄)₂SO₄ supernatant liquid, pH 3.5 supernatant liquid, and pH 3.5 insoluble material contained 3.2, 0.45, and 1.1 mg of protein/ml, respectively, as measured by the method of Lowry et al. (12).
- Initiator assay system, as described in Materials and Methods.
- Lytic assay system of Gould and Hitchins (5).

The initiator in the crude extract was activated by heat (Table 1), but after precipitation with (NH₄)₂SO₄ and dialysis it was apparently heat-sensitive. One explanation of this difference in heat sensitivity is suggested by the experiments of Mishiro and Ochi (16), which indicated that DPA protects serum albumin from heat denaturation. Also, the heat stability of B. subtilis spore glucose dehydrogenase was enhanced with the presence of 0.5 M DPA (7). The heat stability of the initiator in the crude extract is a result of DPA protection; it should be possible to protect the partially purified heat-sensitive initiator against heat inactivation by the addition of DPA. The heat sensitivity of the initiator was decreased by adding DPA (Fig. 3, curve E). DPA did not stimulate initiator activity, but it inhibited slightly (curve B). Either 2 or 5 min at 65 C (curves C and D) significantly decreased initiator activity in the absence of DPA. These results cannot be attributed to an increase in ionic strength because the ionic strength of the DPA-treated sample was actually less than that of the control sample.

To characterize the initiator protein further, the crude extract was fractionated with (NH₄)₂SO₄. The extract (60 ml) was first treated with four nucleases, and then (NH₄)₂SO₄ was added to 100% saturation, as described in Materials and Methods. The precipitate was collected by centrifugation and was resuspended in 5 ml of 0.01 M Tris buffer (pH 8.5), containing (NH₄)₂SO₄ at a concentration that was 65% of saturation. After mixing for 10 min at 25 C, the solution was centrifuged at 10,000 x g for 10 min at 25 C. This sequence was repeated with decreasing concentrations of (NH₄)₂SO₄, as illustrated in Fig. 4. Each of the fractions (about 5 ml) was dialyzed against 1 liter of 0.1 M NaCl-0.01 M Tris (pH 8.5) for 48 hr at 0 C with five changes of buffer. L-Alanine dehydrogenase and adenosine deaminase (which can degrade NAD to inosine), as well as the initiator activity, were assayed in each fraction. The differential fractionation of these enzymes by (NH₄)₂SO₄ is shown in Fig. 5. Fraction C contained 50% of the total initiator activity but was devoid of both L-alanine dehydrogenase and adenosine deaminase activity. The rest of the initiator activity appeared in fraction D, which also contained about 70% of the total amount of L-alanine dehydrogenase, but it lacked adenosine deaminase.
These activity. These results indicated that neither exogenously added adenosine deaminase nor L-alanine dehydrogenase is a component of the initiator system.

Properties of the fractionated initiator. The molecular weight of the initiator was determined in fraction D because this fraction had significant amounts of L-alanine dehydrogenase (molecular weight, 248,000), which may serve as an internal marker. By filtration through Sephadex G-200 (1), a well-defined activity peak for the initiator occurred, which corresponded to a molecular weight of 67,000 (Fig. 6). Because of the extreme lability of the initiator at this stage of purification, further studies on this more purified preparation were not possible. Similarly, the lability of the initiator in fraction C prevented an accurate determination of its molecular weight on Sephadex G-200, but it was estimated to be about 60,000. When the molecular weights for the initiator were established by sedimentation velocity in sucrose gradients by the method of Martin and Ames (13), with bovine serum albumin as a marker, the values in fractions C and D were 74,000 and 71,000, respectively.

Although the mechanism of action of the initiator is not understood, it was independent of both L-alanine dehydrogenase and adenosine deaminase activities, but it was dependent on the presence of two cofactors, NAD and L-alanine. Since fraction C was free from both of the above enzymes, the physical constants for the initiator were determined in this fraction. The interdependence of L-alanine and NAD concentrations during germination with fraction C was investigated (Fig. 7). Varying the amount of L-alanine at different NAD levels caused the slopes in the reciprocal plots to change and at high levels of NAD, the intercepts did not change appreciably. This experiment indicated that, at high concentrations of NAD (6.25 × 10^{-5} to 2.5 × 10^{-4} M), NAD and L-alanine react with the same isomeric form of the initiator; but, at low levels of NAD, the slopes are parabolic, and the apparent intercepts vary, indicating either that L-alanine is acting as an activator for the the initiator (in addition to being a cofactor) or that there is more than one binding site for L-alanine on the initiator.

If NAD is varied at different L-alanine levels, the reciprocal plots are linear, indicating again that NAD and L-alanine probably react with the
Fig. 6. Gel filtration of fraction D. Sephadex G-200 was prepared, and a column (2.5 × 93.5 cm) was constructed, as described in Pharmacia Bulletin No. 6. The column was equilibrated in 0.1 M NaCl-0.01 M tris(hydroxymethyl)aminomethane (Tris) (pH 8.5), at 0°C. The column constants were $V_c = 154$ ml, $V_t = 294$ ml, l-alanine dehydrogenase $K_m = 0.204$, alcohol dehydrogenase $K_m = 0.320$, and cytochrome c $K_m = 0.836$; the flow rate was 0.43 ml/min with a hydrostatic pressure of 20 cm. An amount of 5 ml of fraction D from Fig. 5 was layered on top of the column. The sample was filtered with 0.1 M NaCl-0.01 M Tris (pH 8.5) and 2.0 ml fractions were collected. The absorbancy at 280 nm of alternate fractions (dashed line) was determined on a Beckman DB spectrophotometer. L-Alanine dehydrogenase was assayed with 0.4 ml of the indicated fractions and is plotted as ΔOD/min at 340 nm (C). Initiator activity was assayed with 0.4 ml of the indicated fractions in the presence of L-alanine (10^{-3} M) and nicotinamide adenine dinucleotide (1.25 × 10^{-4} M).

The initiator activity (%) is plotted as change (%) in optical density per 30 min (625 nm) minus background.

Fig. 7. Concentration dependence of the fraction C for l-alanine. The (NH4)2SO4 fraction C (described in Fig. 5) was assayed in the presence of varied amounts of l-alanine at the indicated molar concentrations of nicotinamide adenine dinucleotide. The velocities were calculated from the $t_{50}$ values.

minetetraacetic acid or difluorophosphate as would have been expected if alkaline or neutral proteases were involved in initiator activity. Our data show that only two protein fractions are active; thus the possibility that the initiator activity is serving as a nonspecific protein substrate of some enzyme in the activated spore seemed unlikely. Also, serum albumin or gelatin failed (J. C. Vary, unpublished data) to support initiation of heat-activated spores.

Discussion

In an attempt to explain the statistical behavior of a population of germinating spores, a model has been proposed (9) that predicts the existence of an initiating enzyme for germination. Available data suggest several spore enzymes that may satisfy the role of an initiator.

The enzyme might be l-alanine dehydrogenase, which deaminates l-alanine to pyruvate (8). Although l-alanine dehydrogenase is functional during germination, it may not represent the rate-limiting or primary step in initiation. Mutant spores of B. subtilis that contain little or no l-alanine dehydrogenase retained the ability to germinate with l-alanine (4). L-Alanine dehydrogenase-negative spores germinated slower than the wild type with l-alanine, indicating that the presence of l-alanine dehydrogenase is advantageous to the spore, probably because it provides a readily available energy source from L-alanine. Similarly, the activity of leucine dehy-
hydrogenase, adenosine deaminase, and ribosidase provide methods for utilizing the available energy sources during germination.

Since initiation is degradative, primary candidates for the initiator are hydrolytic enzymes. It was proposed that a protease, subtilopeptidase A, initiated germination (18). However, it was subsequently demonstrated that this apparent proteolytic initiation was caused by the auto-digestion of subtilopeptidase A which resulted in the release of free L-alanine, the actual initiator (6). A muramidase-like enzyme has also been implicated in initiation (5). This lytic enzyme (18) solubilizes the cortex of nonviable spores. Although the cortex probably is solubilized by lytic enzymes during germination, the inability of the Strange and Dark S-enzyme to cause physiological germination (i.e., inability of treated spores to germinate in the usual germinants) indicated that the hydrolysis of the cortex was not the primary initiating event in germination (19). Also it has been impossible to explain the role of initiating agents such as L-alanine by this lytic model.

Another candidate for the initiating enzyme is alanine racemase. Since endogenous D-alanine is released during germination, spores probably have a high ratio of D-alanine to L-alanine. This high ratio might inhibit germination by curtailing the activity of stereospecific enzymes that require L-alanine. Although alanine racemase may be important during sporulation and maintenance of dormancy, it has been shown that, under certain conditions, germination is independent of the activity of that enzyme (2).

On the basis of the properties that one would expect for the "initiating enzyme," the above enzymes are unsatisfactory possibilities for the substance controlling the rate-limiting step in germination.

The experiments reported here demonstrate that dormant spores contain a substance that initiates germination. This substance, which possesses a number of the properties one might predict for a hypothetical initiating enzyme, was inactive in dormant spores but was partially active in heat-activated spores. Moreover, it could be activated in vitro with heat, and it apparently required two factors, L-alanine and NAD. Low concentrations of L-alanine, \( \frac{1}{2} \) of the \( K_m \) for L-alanine-initiated germination, acted catalytically with NAD and the crude extract to initiate germination. Although the mechanism of action is unknown, the initiating substance could gain access to the "site of germination" in heat-activated spores, whereas dormant spores were almost resistant to the initiator. It is possible that dormant spores were impermeable to the initiator and that the structural rearrangements that occurred during heat-activation (17) allowed the entry of the exogenous initiator.

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**LITERATURE CITED**

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