Function and Location of a 'Germination Enzyme' in Spores of *Bacillus cereus*

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SUMMARY

An enzyme extracted from *Bacillus cereus* spores caused to germinate spores of this organism which had been sensitized by reagents which rupture disulphide bonds. Inactivation of the enzyme by thiol-blocking agents and by oxidation, and reactivation by reduction suggested that the enzyme's ability to germinate spores depended on thiol groups. No evidence was obtained to support the hypothesis that the enzyme was present in dormant spores in the oxidized inactive form and became reduced and active during germination. When spores were disrupted at pH 3 the enzyme was found to be bound to debris; probably on or within the central core of the spore. At pH 5.8 or below the enzyme remained bound to the debris, but at pH 7.0 the enzyme was irreversibly released when the ionic strength of the medium was high. In solutions of sodium phosphate less than 0.05 M, the enzyme remained mostly bound even at pH 7.0. It was largely released when the concentration of sodium phosphate was increased to 0.2 M. Extracts of germinated spores contained more unbound and less bound enzyme than extracts of ungerminated spores, suggesting that release of enzyme from a bound form occurred during germination of the spores.

INTRODUCTION

The germination of a bacterial spore involves the change of a stain-resistant phase-bright metabolically-dormant and heat-resistant organism into a readily-stainable phase-dark, metabolically-active and heat-sensitive form. This change can occur in as little as 30 sec. (Vary & Halvorson, 1965). This remarkable 'trigger' reaction can often be initiated by low concentrations of a single amino acid, riboside or sugar. It can also be simulated by what appear to be less natural means; i.e. by mechanical abrasion (Rode & Foster, 1960*a*), by surface active substances (Rode & Foster, 1960*b*), by alkanes (Rode & Foster, 1965), by enzymes like lysozyme (Gould & Hitchins, 1963), a spore enzyme (Gould & Hitchins, 1965) and by a heat-activated factor + a cofactor in *Bacillus cereus* strain τ spore extracts (Vary, 1965). How a 'germinant' initiates the normal germination reaction is unknown. One possible mechanism of germination was suggested when it was found that lysozyme made spores germinate, probably by hydrolysing β 1-4 glycosidic bonds in mucopeptide in the spore cortex (Gould & Hitchins, 1963). To show germination by lysozyme, spores were first treated with reagents which ruptured disulphide bonds: this opened the spore coat structure, presumably by attacking the keratin-like

component of spore coats (Vinter, 1962; Kadota, Iijima & Uchida, 1965), and allowed lysozyme to reach lysozyme-sensitive mucopeptide in the underlying cortex (Gould, Georgala & Hitchins, 1964). Although lysozyme has not been detected in spores, extracts of spores of *B. cereus* contain an enzyme which, like lysozyme, can germinate sensitized *B. cereus* spores (Gould & Hitchins, 1965). Since this enzyme was normally present in *B. cereus* spores, and was able to germinate its spores, it seemed possible that this action within the spore was a prime event in normal germination. Within a dormant spore the enzyme clearly does not attack its substrate; however, on addition of the correct germinant the enzyme may act to cause rapid germination. The theory that germination involved depolymerization of spore mucopeptide by a lytic enzyme was originally proposed by Powell & Strange (1956). The purpose of the experiments described in this paper is to try to find whether activation, or release of the enzyme from a bound form does occur during germination.

METHODS

Production of spores. Spores of *Bacillus cereus*, strain PX were grown and cleaned, as described previously (Hitchins, Gould & Hurst, 1963).

Preparation of spore enzyme. Spore extract containing the enzyme was made by disrupting spores (equiv. 20 mg. dry wt./ml.) in water in a Braun tissue disintegrator (Shandon Scientific Co. Ltd., 6 Cromwell Place, London S.W. 7), whilst cooled to about 4° by a stream of carbon dioxide from a cylinder of liquid CO₂. Debris was then removed by centrifugation at 77,000g for 30 min. at 4°. Supernatant fluid was then used as crude enzyme. This could be partly purified by sequential precipitation in 0.1 M (pH 3.4) buffer (sodium citrate+phosphate) and in 60% saturated (NH₄)₂SO₄ (Gould & Hitchins, 1965).

Assay of spore enzyme. Sensitized spores were made by incubating spores (equiv. 10 mg. dry wt./ml.) in 8 M-urea+25% (w/v) thioglycollic (mercaptoacetic) acid at 70° for 30 min. then washing 6 times with distilled water. Such spores were unable to germinate in the usual germinants (e.g. L-alanine, inosine, yeast extract) and must be presumed non-viable.

The enzyme was assayed by suspending sensitized spores (sufficient to give an extinction of about 0.3 at 580 mμ) in 0.1 M-sodium phosphate buffer (pH 7.0) containing the enzyme (usually 0.5 ml. of crude or partly purified spore extract). Unless otherwise stated, sodium thioglycollate (0.01 M-thioglycollic acid neutralized with NaOH) was included in each assay mixture to maintain the enzyme in a reduced form. The reaction mixture, in a total volume of 5 ml., was incubated at 37° and the extinction read at intervals. The extinction decreased when active enzyme was present. At the same time microscopical examination revealed that active enzyme caused phase-bright spores to become phase-dark. Decrease of extinction and phase darkening are changes characteristic of normal spore germination.

Preparation of spore debris containing cores. Spores in sodium citrate+phosphate buffer (0.1 M) at pH 3 were disrupted in the Braun tissue disintegrator at 4°. During disruption at this low pH value cores were released from within spore coats (Hitchins & Gould, 1964). The resulting core+coat mixture was washed with buffers (0.1 M) at pH 3 then at pH 5.8 by centrifugation, then resuspended in the appropriate buffer (see Results).

Chemicals and buffers. These were of reagent grade. Buffers (0.1 M) were sodium citrate + phosphate (pH 3.0) and sodium phosphate (pH 5.8–8.0). Sodium acetate (pH 5.8 and 7.0) was used whenever calcium chloride was added.

RESULTS

Germinative action of spore extracts

Sensitized spores incubated with spore enzyme became phase dark and shed dipicolinic acid as the extinction of the suspension decreased. The response was most rapid at about pH 7.8 and was similar to the response of spores to a germinant such as L-alanine or inosine (Gould & Hitchins, 1965), however, it occurred with sensitized spores which were non-viable. The enzyme did not cause germination of spores which had not been previously sensitized, in these respects germination induced by the enzyme was similar to germination induced by lysozyme or hydrogen peroxide (Gould & Hitchins, 1963).

Table 1. *Effect of ions on activity of the spore germination enzyme of Bacillus cereus*

Solutions were all adjusted to pH 7.0 with NaOH or HCl and the assay medium contained 0.1 M-sodium phosphate or acetate buffer (see Methods).

Ions added	Concentration (M)	Time for 30 % decrease in extinction of sensitized spore suspension (min.)
NaCl	0.1	26
LiCl	0.1	40
KCl	0.1	33
CsCl	0.1	38
MgCl ₂	0.1	33
CaCl ₂	0.2	120
Chlorides of Ni ²⁺ , Zn ²⁺ , Fe ³⁺ , Co ²⁺	0.1	> 200
Na ethylene diaminetetra-acetate	0.01	21
Na dipicolinate	0.01	30
CaCl ₂ + sodium dipicolinate	Each 0.01	24

Stimulation of enzyme activity by ions

Tris buffer is known to sequester metal ions and it inhibits the enzyme (Gould & Hitchins, 1965). The effects of a number of ions and sequestrants on activity of the enzyme were therefore examined. Various ions (apart from heavy metals), ethylene-diaminetetra-acetate (EDTA), and dipicolinate had small effects on activity of the enzyme (Table 1). Although the nature of the ions was relatively unimportant, their concentration affected the activity of the enzyme, which increased with increasing ionic concentration (Table 2). The enzyme was optimally active when the medium contained about 0.1 M-NaCl, Na₂SO₄ or K₂SO₄ or about 0.2 M-KCl.

Thiol groups and activity of the enzyme

The importance of thiol groups on the enzyme was suggested by its activation by thioglycollate (Gould & Hitchins, 1965). This was confirmed by inhibiting the

enzyme with *p*-chloromercuribenzoate, *N*-ethylmaleimide and Cu^{2+} and by inactivating it by oxidation with iodine or with oxygen (Table 3). Sodium thioglycollate annulled the inhibitions and reactivated enzyme which had been inactivated by oxidation or by thiol-blocking reagents.

Table 2. *Effect of concentration of ions on activity of the spore germination enzyme of Bacillus cereus*

Each solution contained 0.01 M-sodium phosphate and was adjusted to pH 7.0.

Ions added	Concentration (M)	Time for 30 % decrease in extinction of sensitized spore suspension (min.)
NaCl	0.02	> 200
	0.1	29
	0.2	30
KCl	0.02	> 200
	0.1	104
	0.2	45
Na_2SO_4	0.02	> 200
	0.1	27
	0.2	56
K_2SO_4	0.02	> 200
	0.1	28
	0.2	55

Table 3. *Inactivation of the spore germination enzyme by reagents which affect thiol groups*

Sodium thioglycollate was used at 10 mM. Iodine was dissolved in $\times 4$ molar concentration of KI solution. After incubation of enzyme + iodine, excess iodine was removed by adding 82 mM-histidine.

Treatment of enzyme solution	Time for 30 % decrease in extinction of sensitized spore suspension (min.)	
	Thioglycollate absent	Thioglycollate present
No treatment	18	18
Oxygen at 2 atmospheres pressure, 20 min., 37°	87	67
Iodine solution, 30 min. 0°	0.034 mM	30
	0.17 mM	17
<i>p</i> -Chloromercuribenzoate (mM) present during assay	> 200	38
<i>N</i> -Ethylmaleimide (mM) present during assay	> 200	20
CuSO_4 (0.04 mM) present during assay	> 200	Not tested
		56

It seemed possible that the enzyme could be in an inactive oxidized form in spores and become reduced and active during germination. If this were so, extracts of ungerminated spores should contain enzyme in the inactive (oxidized) form. Freshly prepared extracts, however, always contained active enzyme, and the activity decreased during storage. It was still possible that the enzyme was in an inactive form in spores and became reduced and active during breakage, perhaps

because of a reducing action of other enzymes released into the extract. To avoid this, spores were broken at 4° and pH 3.0 or pH 10.6 at which extremes it was hoped that any enzyme activity in the extracts would be minimal. However, similarly active extracts were obtained in each instance, suggesting that the enzyme in dormant spores was already in the active reduced form. When extracts of ungerminated spores were compared with extracts of freshly germinated spores (95 % germinated after 5 min. in yeast glucose broth at 37°), no differences in the amounts of enzyme were found; it appeared that no change of inactive to active enzyme occurred during germination.

Fig. 1. Binding of spore germination enzyme to spore debris at different pH values. Debris was made by breaking spores at pH 3.0 as described in Methods. Samples were then extracted for 15 min. at 4° with 0.2 M-sodium phosphate buffers at the indicated pH values. Enzyme remaining bound to the debris was then released by ultrasonic treatment in sodium phosphate buffer (pH 8.0). The figure shows the extinction decrease of sensitized spores at 37° caused by this enzyme; from pellets extracted at pH 5.8 (●), pH 6.6 (×), pH 7.0 (+), pH 7.8 (■), enzyme-free control (□).

Location of the enzyme in spores

Spores broken at pH 3.0 yielded a mixture of spore coats, cores and insoluble matter (see Methods). After washing at pH 5.8 and resuspending in buffer at pH 5.8 the debris consisted of spore coats and cores free from dipicolinic acid. No enzyme was detected in the supernatant fluids during this procedure, although the isolated enzyme is soluble at pH 5.8. However, when the debris was resuspended at pH 7.0, enzyme was released from it and was detected in the supernatant fluid. When debris was subjected to ultrasonic treatment at pH 7.0 or 8.0 a further small release

of enzyme occurred. Figure 1 shows that enzyme which was bound to the debris at about pH 5.8 was released at higher pH values. This release of enzyme from a bound form was irreversible, i.e. when the pH value was decreased to 5.8 again enzyme was not re-absorbed by the debris during 1 hr at 4°. It therefore appeared that the enzyme was bound to some component of the debris, and that an increase in pH value weakened the binding so that enzyme was released.

Fig. 2. Binding of spore germination enzyme to spore debris in media of low molarity. The figure shows the extinction decrease of sensitized spore suspension at 37° caused by enzyme extracted from spore debris with sodium phosphate solutions (pH 7.0) of different strength: 0.003 M extract (●), 0.2 M extract (○), 0.2 M extract of debris previously extracted with 0.003 M-sodium phosphate (×).

Debris was examined microscopically to find out to which component the enzyme was bound. Preparations at pH values of 3.0, 5.8 and 7.0 all contained fragments of spore coats. However, the preparations differed because at pH 3.0 the cores were phase bright and small (Pl. 1, fig. 1), at pH 5.8 cores were phase dark though still small (Pl. 1, fig. 2) and at pH 7.0 they were phase dark and swollen with indistinct outlines (Pl. 1, fig. 3). Ultrasonic treatment disintegrated most of the cores. When spores were disrupted at pH 5.8 no cores were formed, and the enzyme was not bound to the debris, which consisted of fragments of spore coat, but was found in the supernatant fluid. It seemed most likely that the enzyme was normally bound in or on the spore core, where it remained as long as the pH value did not exceed about pH 5.8. These experiments were made in 0.1 M buffers. When buffers with different molarities were used, the enzyme remained bound to debris even at pH 7.0 when the molarity was low. For example, Fig. 2 shows how the

enzyme was extracted from debris much more efficiently by 0.2 M than by 0.003 M sodium phosphate (pH 7.0). Thus, low pH value and low molarity favoured binding of the enzyme, and high pH value and high molarity caused its release.

Factors affecting binding of enzyme to spore debris

Apart from pH value and ionic strength, other factors affected the binding of enzyme to spore debris. For instance, the polyanionic sequestering agent ethylenediaminetetra-acetate (EDTA) decreased the amount of enzyme liberated in low molarity sodium phosphate, whilst the polyamine spermine caused a greater release (Fig. 3). Inosine, calcium and *n*-dodecylamine (a chemical germinant; Rode & Foster, 1960*b*), did not affect release during 5 min. at 4°. Incorporation of sucrose (15%, w/v) into the solutions did not prevent release of enzyme in 0.2 M-sodium phosphate solution, suggesting that release of enzyme was not due to osmotic lysis of some structure within which the enzyme was normally held.

Fig. 3. Chemicals affecting release of spore germination enzyme from spore debris in media of low molarity. Debris was suspended for 5 min. at 4° in 0.02 M-sodium phosphate (pH 7.0) + the following chemicals: no addition (●), saturated *n*-dodecylamine (○), 4 mM-inosine (×), 17 mM-calcium dipicolinate (⊕), 20 mM-sodium EDTA (■) and 20 mM-spermine hydrochloride. (□) The figure shows that extinction decreased during subsequent assay of enzyme which was released in the presence of the various chemicals.

Binding of enzyme in ungerminated and germinated spores

Spores and germinated spores were compared as sources of the enzyme. It was found that during germination a change in the ratio of enzyme bound to debris in 0.02 M buffer (sodium phosphate, pH 7.6):enzyme free in 0.02 M buffer occurred. Table 4 shows this change in extracts made from ungerminated spores and from spores which were incubated in the germinant inosine until about 95% germination had taken place. The amount of bound enzyme had decreased and the amount of unbound enzyme had increased during germination of the spores.

Table 4. *Free and bound spore germination enzyme in ungerminated and germinated spores of Bacillus cereus*

Spores were germinated by incubation for 6 min. at 37° in 0.1 M-sodium phosphate buffer (pH 8.0) containing 5 mM-inosine. Buffer used to extract enzyme was sodium phosphate pH 7.6.

Source of extract	Time (min.) for 30 % decrease in extinction of sensitized spore suspension during assay of enzyme	
	Subsequently	
	Extracted by 0.02 M-buffer	extracted by 0.2 M-buffer
Ungerminated spores	76	16
Germinated spores	38	31

DISCUSSION

The spore enzyme which will germinate suitably sensitized spores of *Bacillus cereus* is probably similar to spore 'S-enzyme' described by Strange & Dark (1957). This enzyme released soluble mucopeptide from spore walls. It did not lyse the same vegetative bacteria as did lysozyme, but it did attack spore walls and cell walls of some vegetative bacteria (Strange & Dark, 1957). Spore enzymes lysed chloroform-treated *Escherichia coli* (Work, 1959, Gould, 1962) suggesting that these enzymes attacked mucopeptide, although with a specificity different from lysozyme. The exact substrate(s) of spore lytic enzymes have not, however, been determined.

The fact that the enzyme in *Bacillus cereus* spore extracts can induce in sensitized *B. cereus* spores the changes which are normally recognized as germination, suggests that it might well have a primary role in germination. This is also suggested by the optimal germinative activity of the enzyme in media of ionic strength equivalent to about 0.1 M-NaCl, for Fleming & Ordal (1964) found that normal germination was also most rapid in media of about this ionic strength. Furthermore, removal of ions from the media inhibited germinative action of the spore enzyme, and is also known to inhibit normal spore germination (Rode & Foster, 1962).

Studies on the activation of spores by heat (Busta & Ordal, 1964) and by reducing agents (Keynan, Evenchik, Halvorson & Hastings, 1964) suggested that in spores which normally require some such form of activation in order subsequently to germinate rapidly, rupture of disulphide bonds in spore enzymes might be part of the activation process. It was therefore of interest to find that the *Bacillus cereus* enzyme studied in the present work was inactivated by oxidation and subsequently reactivated by reduction. Experiments to find in which form the enzyme existed in the ungerminated spore, however, all suggested that this was in the active reduced form, and therefore the enzyme did not require activation by reduction in order to attack its substrate.

Another method by which the enzyme within a spore might conceivably be made to attack its substrate during germination would be by release from a bound form (like lysosomal enzymes). It was therefore of interest to find that the enzyme was bound to spore debris, most probably to some part of the spore core. In our experiments, the enzyme was easily released from its bound form by increasing the pH

value or by raising the solute concentration. The only chemical tested which effectively caused release of the enzyme was the basic polyamine spermine which has strong affinity for electronegative structures, e.g. cell membranes (Mager, 1959). Spermine therefore probably released the enzyme by displacing it from its attachment to electronegative groups on or within the core. The ease with which enzyme was released by the various treatments suggests that its attachment is by weak electrostatic bonds or salt linkages and not by covalent bonding. Failure of EDTA to effect release of enzyme makes a role for calcium in linking enzyme to debris seem unlikely.

In an intact spore could some enzyme release mechanism operate during germination? If a germinant (e.g., L-alanine, inosine), or some product of its metabolism in a spore, initiated the release, then germination would quickly occur as freed enzyme attacked its substrate. The observation that during inosine-induced germination of spores an increase in the ratio of free:bound enzyme occurred, suggests that such a release of enzyme did take place. The substrate of the enzyme is probably the structural material of the spore cortex, which is predominantly mucopeptide (Warth, Ohye & Murrell, 1963). The importance of this mucopeptide in maintaining the dormant resistant structure of spores of various bacillus and clostridium species was emphasized by the observation of the germinative action of lysozyme (Gould & Hitchins, 1963), because lysozyme is known to hydrolyse mucopeptide. A search for an enzyme-release mechanism which could be initiated by a germinant or some product of its metabolism by a spore, will clearly be worthwhile. Apart from the possible role of the spore enzyme in germination, fractionation of spores and probable location of the enzyme in one structure (i.e. the core) is of interest. For instance, most enzymes in spores are somehow rendered not only heat-resistant but also dormant, i.e. they do not act upon added substrates. However, the spore enzymes which metabolize germinants cannot be dormant; they must be capable of acting on their substrates in the otherwise dormant spore in order to initiate germination. Further study should reveal whether enzymes, such as L-alanine dehydrogenase, alanine racemase, adenosine deaminase, ribosidase, etc., which one would expect to have a role in metabolizing germinants, are located in different regions of the spore to enzymes concerned with outgrowth of new vegetative cells.

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EXPLANATION OF PLATE

Phase-contrast micrographs of debris from spores of *Bacillus cereus* which have been disrupted at pH 3.0. The scale mark represents 5 μ .

Fig. 1. Debris in pH 3.0 buffer. The figure shows one unbroken phase-bright spore (*s*) and fragments of spore walls (*w*). A group of cores (*c*) liberated from within spore walls are phase-bright at pH 3.0.

Fig. 2. Debris in pH 5.8 buffer. An unbroken spore (*s*) and spore walls (*w*) appear as in Fig. 1. Cores (*c*) appear phase-dark at pH 5.8.

Fig. 3. Debris in pH 7.0 buffer. Cores (*c*) have now swollen and become pale and diffuse in outline.

Fig. 1

Fig. 2

Fig. 3