Zinc release and the sequence of biochemical events during triggering of *Bacillus megaterium* KM spore germination

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Zinc release is the first quantitatively significant event detected during the triggering of *Bacillus megaterium* KM spore germination. Of the total spore Zn^{2+} pool 25% is released from non-heat-activated spores within 4 min of triggering germination. During this period only 10% of the spore population becomes irreversibly committed to germinate. The investigation of a putative role for Zn^{2+} in the germination trigger mechanism has established a relationship between the rate and extent of Zn^{2+} release and the stimulation of spore germination by heat activation. Furthermore, a correlation can be demonstrated between the extent of zinc release from spore populations and the time required to obtain 50% commitment of these populations to germinate over a wide temperature range. These findings have been used to expand a recently published model for the triggering of bacterial spore germination.

Determinations of the rate of commitment of bacterial spores to germinate after short exposure to L-alanine have prompted the development of a model to explain the phenomenon of commitment in terms of a trigger event (Stewart *et al.*, 1981). This model was unable to suggest a mechanism whereby the spore could generate the critical concentration of an active allosteric receptor protein required for the commitment reaction and hence subsequent germination to proceed.

Triggering of *Bacillus megaterium* KM spore germination takes place in the absence of germinant metabolism or germinant-stimulated metabolism (Scott & Ellar, 1978*a,b*). The lag period between the rapid commitment event and the onset of general spore metabolism in this organism permits the analysis of the biochemical changes that take place during the trigger reaction. Thus, it has recently been demonstrated that, as part of the trigger reaction, the spore responds to germinants by initiating the activity of latent enzymes capable of reducing cortex rigidity by selective hydrolysis (Johnstone & Ellar, 1982). Although a cortex-lytic enzyme could be the functional role of the model allosteric receptor protein, such a direct relationship has not been established. Further characterization of any such

relationship requires the identification and study of other biochemical changes that are associated with the trigger event.

The influence of ions and ion flux on metabolite-induced germination has been extensively studied (Rode & Foster, 1962*a,b,c*; Foerster & Foster, 1966; Rode & Foster, 1966; Crosby *et al.*, 1971) and any theory relating to the mechanism of germination should take account of the well-documented dependence on the ionic environment (Rode & Foster, 1962*a*). Previous studies, however, have not measured the ion flux during germination on a time scale that would be pertinent to an examination of the trigger event (i.e., 0–5 min after addition of germinants). The present paper describes experiments that were carried out to detect ionic changes over this time scale and to examine the relationship of any such changes to the phenomenon of heat activation, which has recently been demonstrated to stimulate the rate of the trigger reaction (Stewart *et al.*, 1981) in addition to the overall rate of germination (Keynan & Evenchik, 1969).

Materials and methods

Spore preparation and germination

Spores of *B. megaterium* KM were prepared as described previously (Stewart *et al.*, 1981). Germination was measured by the loss of absorbance of spore suspensions at 600 nm (Scott & Ellar, 1978*a*). For heat-activation studies, spores were suspended

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at 10mg dry wt./ml in deionized water and incubated at 70°C until cooled on ice immediately before germination.

In certain experiments spores were grown in CCY medium (Stewart *et al.*, 1981) containing $^{65}\text{Zn}^{2+}$ (330 MBq/mmol; 0.05 mM).

Determination of pyridine-2,6-dicarboxylic acid and metal ions

In order to measure ion release during heat activation and germination, spore suspensions were rapidly filtered as previously described (Scott & Ellar, 1978c). Pyridine-2,6-dicarboxylic acid was determined in heat-activation and germination filtrates by using the difference-spectrum assay of Scott & Ellar (1978c). The metal ion content of filtrates was measured by using a Unicam SP90 Atomic Absorbance Spectrophotometer including internal standards where necessary to determine the degree of quenching. The metal ion content of spores was measured by ashing spores at 500°C for 1 h in Pt crucibles. The residue was dissolved and diluted in 0.1 M-HCl and the metal ion content was determined as described above. The release of Zn^{2+} from spores prepared in medium containing $^{65}\text{Zn}^{2+}$ was determined by scintillation counting of filtrate samples as previously described for $^{45}\text{Ca}^{2+}$ (Scott & Ellar, 1978c).

Results and discussion

In order to examine the nature and extent of ion flux during the trigger reaction, the release of ions during the first minutes of germination of non-heat-activated spores was determined. The results shown in Fig. 1 demonstrate that release of Ca^{2+} , pyridine-2,6-dicarboxylic acid and Zn^{2+} commences immediately on addition of germinants. Furthermore, when considered in terms of total spore ion composition, 25% of the total spore Zn^{2+} pool is released in the first 4 min of germination, compared with less than 2% of the spore Ca^{2+} or pyridine-2,6-dicarboxylic acid. Thus during the time period required to obtain 10% commitment of non-heat-activated spores (Stewart *et al.*, 1981), 25% of the total Zn^{2+} pool is mobilized and released.

The earliest spore response to germinants detectable in our laboratory is the binding of the germinant L-alanine, which reaches saturation for both heat-activated and non-heat-activated spores within 2 min at 30°C (M. A. Konciewicz, personal communication). Subsequently, Zn^{2+} release is the first quantitatively significant event and this strongly suggests that Zn^{2+} displacement may represent the initial biochemical event of the L-alanine-stimulated trigger reaction.

Further investigation of this involvement of Zn^{2+} requires a means of perturbing the trigger reaction

Fig. 1. The release of ions during spore germination. Non-heat-activated spores were germinated at 10mg/ml in 50mM-KCl and 1mM-L-alanine. The release of ions was determined as described above. Symbols: ●, pyridine-2,6-dicarboxylic acid; ○, Ca^{2+} ; □, Zn^{2+} ; △, Mg^{2+} ; ◇, Mn^{2+} .

and subsequently correlating this perturbation with Zn^{2+} movement. Heat activation of bacterial spores has been shown to stimulate the rate of commitment to germinate by a factor of 3 (Stewart *et al.*, 1981). Since this implies that the trigger mechanism in itself must respond to heat activation, it offers an experimental means for perturbing the trigger mechanism. For this reason the potential involvement of ions and in particular Zn^{2+} was investigated during the process of heat activation.

Fig. 2 shows the kinetics of heat-activation expressed as the change in T_{50} (the time required to obtain 50% of the maximum change in A_{600} after

Fig. 2. The effect of heat activation at 70°C on the time required to obtain 50% of the maximum change in A_{600} after initiation of germination at 30°C (T_{50})

Spores were heat-activated and germinated as described in the legend to Fig. 1.

initiation of germination at 30°C) with increasing time of heat-activation at 70°C. A very rapid change was observed within the first 15 min of activation, which was sufficient to account for the degree of stimulation previously described (Stewart *et al.*, 1981). This stimulation may reflect an allosteric activation of the germination trigger mechanism. Longer periods of heat-activation at 70°C resulted in a continued decrease in the T_{50} values, which exponentially approached the limiting value of 4 min [a plot of $\log(T_{50}-4)$ versus time of activation at 70°C gives a correlation coefficient of 0.98].

The profile of ion release from spores during the period of activation described above is shown in Fig. 3. The release of pyridine-2,6-dicarboxylic acid, as the potassium salt (results not shown), was relatively linear with time of activation. The only bivalent metal ion released during activation for up to 6 h was Zn^{2+} and the release of this ion commenced from $t_{0.75}$, which is after the initial rapid activation and co-ordinates with the exponential increase in T_{50} values described above. Crosby *et al.* (1971) have previously reported that Zn^{2+} is the only bivalent metal ion released during heating of *B. megaterium* A.T.C.C. 19213 at 60°C. Other studies have shown

Fig. 3. The release of spore ions during heat-activation in deionized water

Symbols: ●, pyridine-2,6-dicarboxylic acid; △, Zn^{2+} ; □, Ca^{2+} , Mg^{2+} and Mn^{2+} .

that Zn^{2+} is inhibitory when added to germination media at concentrations of 1 mM or above in both *B. megaterium* A.T.T.C. 19213 (Crosby *et al.*, 1971) and *B. megaterium* KM (results not shown), and since Zn^{2+} release during extended heat activation is correlated with an increase in the rate of subsequent germination, these data support a role for Zn^{2+} displacement in the trigger reaction.

The possible involvement of Zn^{2+} in the trigger reaction was further investigated by measuring the Zn^{2+} released from heat-activated $^{65}Zn^{2+}$ -labelled spores during the time required to obtain 50% commitment to germinate over a range of temperatures. The response of the rate of commitment to germination temperature has been described previously (Stewart *et al.*, 1981). The following values represent the time in minutes for 50% commitment at 15, 20, 25, 30 and 35°C respectively: 17.3, 8.16, 3.73, 1.78 and 0.95. Fig. 4 shows an Arrhenius plot obtained from these measurements and from which an activation energy, for the amount of Zn^{2+} released in the time required to obtain 50% commitment (C_{50}), of 1.27×10^5 J/mol can be calculated. This value is very close to that obtained from the commitment reaction itself of 1.08×10^5 J/mol (Stewart *et al.*, 1981) and it is possible that Zn^{2+} release and commitment to germinate may be a measure of the same event. The activation energy obtained from such Arrhenius plots must, however, be interpreted with caution, since it is possible that it reflects a complex series of reactions rather than a single reaction profile.

In order to relate Zn^{2+} release to other events occurring during the first minutes of germination, we have correlated this release with previously published data (Johnstone & Ellar, 1982). Fig. 5 demonstrates that the sequence of events that occurs during the triggering of germination is: Zn^{2+} release, commitment, cortex hydrolysis, pyridine-2,6-di-

Fig. 4. Arrhenius plot of the amount of Zn²⁺ released in the time required to obtain 50% commitment (C₅₀) minus that released during heat-activation versus the reciprocal of absolute temperature

The apparent activation energy (μ) was calculated from the equation $-\mu = \text{slope} \times 2.303 R$ where R is the gas constant ($8.314 \text{ J} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$).

carboxylic acid (and Ca²⁺) release and finally the onset of metabolism, as reflected by irreversible ³H incorporation and net ATP synthesis.

From these observations it is possible to extend the model recently described to explain the trigger event (Stewart *et al.*, 1981).

R is still considered as an allosteric receptor protein that can interact with L-alanine (Ala) to produce AR*. The production of AR*, however, also requires the release of Zn²⁺ from a regulatory site on the receptor protein. We believe that in the non-heat-activated spore the protein exists predominantly in an inactive state P* that cannot interact with L-alanine or release Zn²⁺. Normal heat

Fig. 5. The sequence of events during the triggering of *B. megaterium* KM spore germination by L-alanine

Data are taken from Fig. 1, and the results of Stewart *et al.* (1981) and Johnstone & Ellar (1982). Symbols: ●, Zn²⁺ release (%); □, commitment (%); ■, cortex hydrolysis (%); ○, pyridine-2,6-dicarboxylic acid release (%); ▲, irreversible ³H incorporation (nmol/mg of spores); △, ATP pool (pmol/mg of spores).

activation (70°C/30 min) promotes the conformational change from P to R and extended heat activation (70°C/0.75–8 h) promotes the release of Zn²⁺ from R to yield R*. It is possible that a sufficient concentration of R* may lead directly to germination. However, the system normally requires interaction with L-alanine. In the absence of extended heat activation, interaction of R with L-alanine produces AR, which, possibly through an allosteric change, causes the release of Zn²⁺ to produce AR*. (Clearly, interaction of R with D-alanine, a potent germination inhibitor, must inhibit Zn²⁺ release,

possibly by an aberrant conformational change or the inhibition of a conformational change.) It seems possible that the enzyme reaction catalysed by AR* is cortex hydrolysis, although at present the proteolytic activation of, for example, a cortex-hydrolysing enzyme cannot be excluded.

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