The use of inhibitors to identify early events during *Bacillus megaterium* KM spore germination

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The germination response of spores of *Bacillus megaterium* KM, as measured by loss of A_{600} , is more than 95% inhibited by 1 mM-HgCl₂. Two Hg²⁺-sensitive sites (referred to as 'sites I and II') have been identified during germination. Site I represents a pre-commitment event and can be protected from HgCl₂ by 50 mM-D-alanine, whereas site II represents a post-commitment event and is not D-alanine-protectable. At 1 mM-HgCl₂, 25% of the spore population becomes committed to germinate, but an A_{600} loss of less than 5% occurs. In this system, loss of heat resistance was associated with commitment, whereas selective cortex hydrolysis, release of pyridine-2,6-dicarboxylic acid, Zn²⁺ and soluble peptidoglycan, as well as loss of refractility, were identified as post-commitment events. The commitment event was reversibly inhibited by several proteinase inhibitors and a membrane bulking agent. A model of spore germination based on these results is presented.

INTRODUCTION

Exposure of spores of *Bacillus megaterium* to the spore-specific germinant L-alanine results in a rapid loss of typical spore properties (Gould, 1969). The accompanying biochemical changes, detectable within minutes after L-alanine addition, include release of Zn²⁺, selective hydrolysis of cortical peptidoglycan, release of calcium dipicolinate and the onset of general spore metabolism (Johnstone *et al.*, 1982). This germination response is mediated by a germinant receptor that initiates a trigger reaction, thereby committing the spore to germinate (Stewart *et al.*, 1981). The mechanism of the triggering and commitment reactions are unknown, but take place in the absence of both germinant (Scott & Ellar, 1978*b*) and germinant-stimulated metabolism (Scott & Ellar, 1978*a*). There is evidence both for inner-membrane (Skomurski *et al.*, 1983; Racine *et al.*, 1981) and for integument (Jenkinson & Lord, 1983) locations for the germinant receptor protein.

Determination of the sequential interrelationships between the early germination events is essential for an understanding of the mechanism of the trigger reaction in relation to the overall germination process. Inhibitors have been widely used to probe the germination pathway (Vinter, 1970; Smoot & Pierson, 1982), which proceeds normally in the presence of a wide range of metabolic inhibitors (Scott & Ellar, 1978*b*). Specific-inhibitor studies have suggested the involvement of a proteinase early in the germination sequence (Boschwitz *et al.*, 1983, 1985). Hg²⁺ ions act as a potent inhibitor of spore germination (Levinson & Hyatt, 1966) and their effects can be reversed by thiol reagents, including thioglycollate and 2-mercaptoethanol (Labbe *et al.*, 1981). Treatment of spores of *B. megaterium* QMB 1551 with 1 mM-HgCl₂ blocked subsequent amino acid uptake, but allowed release of dipicolinic acid and partial loss of refractility (Rossignol & Vary, 1978). At 4 mM-HgCl₂, the germina-

tion pathway was completely inhibited, which suggested that there are two Hg²⁺-sensitive sites during germination.

The present paper describes experiments that were carried out to identify the sites of Hg²⁺ inhibition in relation to the biochemical and physiological events that occur during *B. megaterium* KM spore germination.

MATERIALS AND METHODS

Spore preparation and germination

Spores of *Bacillus megaterium* KM were prepared as described previously (Stewart *et al.*, 1981) and stored at a concentration of 10 mg dry wt./ml in distilled water at –20 °C. Before germination, spores were heat-activated at 70 °C for 30 min and cooled on ice. Germination was initiated by addition of a final concentration of 1 mM-L-alanine to a spore suspension (0.05 mg dry wt./ml) in 50 mM-potassium phosphate buffer, pH 7.0, at 30 °C. The extent of germination was determined as the percentage loss in absorbance at 600 nm of spore suspensions after 40 min (Scott & Ellar, 1978*a*) in a Pye-Unicam SP.8-200 UV-VIS spectrophotometer fitted with a constant-temperature cell holder. The control germination response represents that of untreated spores in the above germination system, for which a 65–70% loss of initial A_{600} was observed.

Measurement of early germination events

Zn²⁺ release was measured by the method of Johnstone *et al.* (1982) with a Baird (Braintree, Essex, U.K.) atomic absorption spectrophotometer. Dipicolinic acid and hexosamine release were measured by the methods of Scott & Ellar (1978*c*) and Ghuysen *et al.* (1966) respectively. Hg²⁺ ions were removed from the hexosamine assay by the addition of final concentrations of 16 mM-dithiothreitol and 5% (w/v) trichloroacetic acid

Abbreviations used: DPA, pyridine-2,6-dicarboxylic acid; NEM, *N*-ethylmaleimide; PMSF, phenylmethanesulphonyl fluoride; Tos-Arg-OMe, *N*^ε-*p*-tosyl-L-arginine methyl ester ('TAME'); Tos-Lys-CH₂Cl, *N*^ε-*p*-tosyl-L-lysylchloromethane ['TLCK' ('tosyl-lysine chloromethyl ketone')].

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(Hsieh & Vary, 1975). Limited cortex hydrolysis was measured as described by Johnstone & Ellar (1982). For these assays, spores were germinated at a concentration of 0.5 mg dry wt./ml in 50 mM-KCl containing 1 mM-L-alanine. HgCl_2 was added from a 24 mM stock solution titrated to pH 6.1. These conditions did not effect the HgCl_2 -sensitivity of the spores. Loss of heat- and u.v.-resistance were measured 15 min after initiation of germination by either heat treatment at 70 °C for 30 min or exposure to u.v. light (sufficient to give > 99.9% decrease in the vegetative-cell count), followed by spread-plate cell counts on thioglycollate medium (Levinson & Hyatt, 1966).

Protection of the germinant receptor protein

Portions (10 ml) of heat-activated spores at a concentration of 0.05 mg dry wt./ml in Decon-washed Corex tubes were incubated for 5 min at 30 °C in the presence of 10–50 mM-D-alanine in 50 mM-potassium phosphate buffer, pH 7.0. After addition of 1–1.75 mM- HgCl_2 , the spore suspensions were incubated for a further 2 min and washed three times by centrifugation (5000 g, 5 min, 4 °C) in 10 ml of 50 mM-potassium phosphate buffer, pH 7.0, with the addition of 10 mM-D-alanine (final concn.) to the first wash. Finally the spores were resuspended in 20 μl of distilled water and germinated as described above. At 15 min after addition of L-alanine, 10 mM-D-alanine (final concn.) was added, followed 1 min later by 6 mM-2-mercaptoethanol (final concn.). The percentage A_{600} loss (compared with that of control spores, which were treated identically in the absence of HgCl_2) was calculated between the time of addition of L-alanine until 40 min after addition of 2-mercaptoethanol.

Inhibition of germination by acetic anhydride

Before acetic anhydride treatment, spores were permeabilized, at a concentration of 10 mg dry wt./ml, in 0.1 M-dithiothreitol/0.1 M-NaCl, pH 10.0, for 30 min at 30 °C, washed five times in distilled water (4 °C) and heat-shocked as described above. Spores were treated, at a concentration of 0.5 mg dry wt./ml, with acetic anhydride for 30 min at 30 °C as described by Rossignol & Vary (1979), washed three times in distilled water (4 °C) and germinated as described above.

Chemicals

D-Alanine, L-alanine, 2-mercaptoethanol, *N*-acetyl-D-glucosamine, DPA, acetic anhydride, PMSF, NEM, Tos-Lys- CH_2Cl , Tos-Arg-OMe, procaine and dithiothreitol were all obtained from Sigma. HgCl_2 (AR grade) and ZnCl_2 (atomic-absorption-spectroscopy standard) were from BDH Biochemicals. NaB^3H_4 (5 Ci/mol) was from Amersham International. All other chemicals were of analytical reagent grade.

RESULTS

Effect of HgCl_2 on the overall germination response

The effect of HgCl_2 on the overall germination response of *B. megaterium* KM spores (as measured by A_{600} loss) was first established. Inhibition of germination by pre-treatment with HgCl_2 showed a biphasic titration, with 50% inhibition at 0.05 mM- HgCl_2 , and more than 95% inhibition at concentrations of HgCl_2 in excess of 1 mM (Fig. 1). Addition of 1.5 mM- HgCl_2 at any point

Fig. 1. Inhibition by, and reversal of the effect of, HgCl_2 on the overall germination response

●, Inhibition of germination by HgCl_2 : spores of *B. megaterium* KM were pretreated with various concentrations of HgCl_2 for 15 min before addition of L-alanine. The percentage inhibition of A_{600} loss 40 min after addition of L-alanine was determined as described in the Materials and methods section. ○, Reversal of HgCl_2 inhibition by 2-mercaptoethanol: spores were treated with HgCl_2 (as above) followed by 6 mM-2-mercaptoethanol for 1 min before addition of L-alanine. The percentage inhibition of A_{600} loss was measured as described above.

during germination subsequently halted the germination process, as reported by Hsieh & Vary (1975) (results not shown). The inhibition of germination of spores pretreated with HgCl_2 could be reversed by 2-mercaptoethanol, and 6 mM-2-mercaptoethanol restored the germination response of spores treated with 1.25 mM- HgCl_2 by 95% (Fig. 1). The effect of 6 mM-2-mercaptoethanol itself on germination was minimal (results not shown). In the presence of 1 mM- HgCl_2 , when loss of A_{600} is more than 95% inhibited, approx. 25% of the spore population becomes committed to germinate (Fig. 2). The rate of commitment observed is less than that for untreated spores (Fig. 2), reaching its maximum rate after a lag period of 4 min and a maximum extent 15 min after addition of L-alanine.

Identification of HgCl_2 -sensitive sites during germination

Commitment to germination represents a defined point in the germination sequence after which germination will proceed normally if the germinants are removed either by washing or by the addition of high concentrations of the competitive germination inhibitor D-alanine (Stewart *et al.*, 1981). The effect of HgCl_2 on spores that had been exposed to L-alanine for 4 min before the addition of D-alanine was therefore examined in order to test for a post-commitment HgCl_2 -sensitive site. At this

Fig. 2. Effect of 1 mM-HgCl₂ on commitment to germination

○, Percentage commitment to germinate determined as described by Stewart *et al.* (1981); ●, percentage commitment to germinate in the presence of 1 mM-HgCl₂: spores were pretreated for 15 min with 1 mM-HgCl₂ before the addition of 1 mM-L-alanine. D-Alanine (10 mM) was added at the times indicated, followed 1 min later by 2-mercaptoethanol to a final concentration of 6 mM. The percentage inhibition of A_{600} loss was determined as for Fig. 1, 40 min after addition of 2-mercaptoethanol.

time, 60% of the spore population has become committed to germinate (Fig. 2) and less than 5% of the original A_{600} of the spore suspension has been lost. A post-commitment site (referred to as 'site II'), which was 98% inhibited by 3 mM-HgCl₂, was observed (Fig. 3). Titration of site II with HgCl₂ also showed a biphasic response, with 50% inhibition at 0.05 mM-HgCl₂. The inhibitory effect of 1.5 mM-HgCl₂ on this site could be 95% reversed by subsequent addition of 6 mM-2-mercaptoethanol (results not shown).

Identification of a reversible post-commitment HgCl₂-sensitive site then allowed analysis for the presence of a similar pre-commitment site. Spores were treated with HgCl₂, followed by the addition of 1 mM-L-alanine. After a further 15 min, 10 mM-D-alanine was added to the spore suspension, by which time more than 95% commitment is normally achieved (Fig. 2). Sufficient 2-mercaptoethanol was added to reverse the inhibition of site II and thus to allow the committed spore population to germinate. At the HgCl₂ concentrations used, the A_{600} loss before 2-mercaptoethanol treatment was less than 5%. The percentage inhibition of subsequent germination (Fig. 3) demonstrates the presence of a pre-commitment HgCl₂-sensitive site (referred to as 'site I') that is 95% inhibited by 2 mM-HgCl₂. Titration of site I shows a characteristic response when compared with either the overall germination titration curve (Fig. 1) or the site-II titration curve (Fig. 3).

Fig. 3. Identification of pre- and post-commitment HgCl₂-sensitive sites

○, Effect of HgCl₂ on a post-commitment site (site II): spores were germinated for 4 min and D-alanine was added to a final concentration of 10 mM. The spores were treated with HgCl₂ 1 min later and the percentage inhibition of A_{600} loss 40 min after initiation of germination determined as for Fig. 1. ●, Effect of HgCl₂ on a pre-commitment site (site I): spores were treated as described for Fig. 2 (●), the concentration of HgCl₂ being as shown; D-alanine was added 15 min after L-alanine in all cases.

Effect of inhibitors on early germination events

In order to relate the sites I and II to other germination events, the effect of HgCl₂ on other early biochemical events was also studied. Zn²⁺, DPA and hexosamine release were more than 95% inhibited by 1 mM-HgCl₂ (Fig. 4). Limited cortex hydrolysis and loss of u.v. resistance were also found to be more than 95% inhibited by 1 mM-HgCl₂ (results not shown). In comparison, 30 ± 5% of the spore population lost heat resistance after 15 min germination in the presence of 1 mM-HgCl₂. In this system, since 25–30% of the spore population has undergone the commitment reaction, it is concluded that loss of heat resistance is the only germination parameter measured that occurs as part of the commitment event.

In common with the findings of Boschwitz *et al.* (1983) for *Bacillus cereus*, several proteinase inhibitors were found to inhibit reversibly *B. megaterium* KM spore germination. Spore germination as measured by A_{600} loss was more than 90% inhibited by 15 min pretreatment of spores with NEM (15 mM), PMSF (3.33 mM), Tos-Lys-CH₂Cl (18 mM) and Tos-Arg-OMe (30 mM). Tos-Arg-OMe (30 mM) also immediately halted A_{600} loss when added to committed spores, whereas the other compounds inhibited A_{600} loss by less than 20%. These concentrations of PMSF and Tos-Arg-OMe both completely inhibited

Fig. 4. Effect of HgCl₂ on early germination events

Spores were germinated and assays performed as described in the Materials and methods section. ○, Percentage inhibition of Zn²⁺ release; △, percentage inhibition of DPA release; ●, percentage inhibition of hexosamine release.

DPA loss and loss of heat resistance by 95 and 81% respectively. Pre-exposure of spores to these concentrations of PMSF, NEM and Tos-Lys-CH₂Cl in all cases inhibited commitment by L-alanine by more than 90% (over a 15 min period) as measured by subsequent loss of *A*₆₀₀ after washing and resuspension. Less than 10% of *A*₆₀₀ (of control) was lost until addition of L-alanine to the washed spores. Procaine (0.4%), a membrane bulking agent (Lazdunski *et al.*, 1979), also had a similar effect on commitment when spores were treated as above.

Acetic anhydride has been shown to be a possible label of the germinant receptor protein in *Bacillus megaterium* QMB 1551 (Rossignol & Vary, 1979). When permeabilized spores at a concentration of 0.5 mg dry wt./ml were treated with 0.5% acetic anhydride, the subsequent germination response, as measured by *A*₆₀₀ loss, was irreversibly inhibited by 100%. This concentration of acetic anhydride also inhibited DPA loss by 90%.

'Protection' of HgCl₂-sensitive sites by D-alanine

The possibility that HgCl₂ inhibition at site I is due to interaction with the spore L-alanine receptor site was investigated by a 'protection' experiment. Spores were incubated with D-alanine before addition of HgCl₂ and then treated as described in the Materials and methods section to allow the committed spore population to germinate. The *A*₆₀₀ was measured over a 56 min period, as some *A*₆₀₀ was lost before addition of 2-mercaptoethanol owing to site II being partially reversible by washing. The presence of 50 mM-D-alanine was able to protect site I fully against 1.75 mM-HgCl₂, whereas

Fig. 5. Protection of site I from the effect of HgCl₂ by D-alanine

Spores were treated and germinated as described in the Materials and methods section. ●, Pretreatment with 50 mM-D-alanine before addition of HgCl₂; ○, pretreatment with 10 mM-D-alanine before addition of HgCl₂; △, no pretreatment with D-alanine before addition of HgCl₂.

10 mM-D-alanine only protects site I from 1 mM-HgCl₂ by 70% (Fig. 5). After protection, 1.25 mM-HgCl₂ caused 83% inhibition (results not shown) when spores were treated as for the site-I titration (Fig. 3). In contrast, site II is not protected by the presence of D-alanine, since under the experimental conditions described for Fig. 3 (site-II titration), spores are pretreated with 10 mM-D-alanine during HgCl₂ treatment.

DISCUSSION

The experiments described in the present study have characterized both pre- and post-commitment HgCl₂-sensitive sites for the first time in spores of *B. megaterium* KM. The overall inhibition of germination by HgCl₂ cannot be completely accounted for by the additive effects of inhibition of sites I and II (Figs. 1 and 3). The presence of a third HgCl₂-sensitive site as a post-commitment, but pre-site-II, event thus cannot be discounted. Alternatively, this effect may be due to the different exposure times of spores to HgCl₂ in the two systems or to an increase in sensitivity of site II to HgCl₂ observed as germination proceeds (results not shown).

Modification of the L-alanine-binding site is the most likely reason for site-I inhibition, since protection by D-alanine is observed. Loss of *A*₆₀₀ is prevented until site II is reversed, and therefore the site-II event is probably associated with degradation of structures that maintain spore refractility. Site II may therefore represent the inhibition of a cortex-lytic enzyme, since the spore cortex is implicated in the maintenance of spore dormancy

(Ellar, 1978) and cortex-lytic enzymes are intricately associated with early germination events (Brown *et al.*, 1977; Johnstone & Ellar, 1982). The observation that HgCl_2 is a potent inhibitor of peptidoglycan autolysis (Peetz & Kenny, 1978) also supports this view.

Characterization of sites I and II may allow two fundamental questions about *B. megaterium* KM spore germination to be answered. Firstly the observation that D-alanine can protect site I from HgCl_2 may allow the germination receptor complex to be identified. This could be achieved by treating spores with non-radioactive HgCl_2 in the presence of D-alanine to label the non-specific Hg^{2+} -binding sites. After washing and incubation with $^{203}\text{HgCl}_2$, spore fractionation may allow the receptor protein to be located and identified. To complement this, treatment of spores with radioactive acetic anhydride may also label the L-alanine binding site. A 10.2 kDa inner-membrane protein has been identified as the possible germinant receptor protein after acetic anhydride and germinant-analogue treatment of spores and vegetative cells of *B. megaterium* QMB1551 (Racine *et al.*, 1981; Rossignol & Vary, 1979). More recently, cloning experiments have suggested that the *B. subtilis* L-alanine germination receptor is transcribed from the *gerA* operon containing three complementation groups (Zuberi *et al.*, 1985). It will be of considerable interest to establish how similar the *B. megaterium* KM L-alanine receptor is to these two systems.

The second question fundamental to our understanding of the germination process is to define its sequence of events. Analysis of biochemical changes during germination have suggested that, in *B. megaterium* KM, L-alanine binding is followed by Zn^{2+} release, selective cortex hydrolysis, commitment and release of Ca^{2+} and dipicolinic acid (Johnstone *et al.*, 1982). The heterogeneity of the spore population and relative insensitivity of certain biochemical assays make it difficult to define which of these biochemical events are associated with the trigger reaction. The difference in sensitivity of the two HgCl_2 -sensitive sites, however, allows germination events to be classed as pre- or post-commitment. Of all the events examined, only loss of heat resistance was found to occur pre-commitment. It is noteworthy that this loss of heat resistance is probably not due to the collapse of the spore structures that maintain the dormant state, since a 25% loss of viability during heat treatment of committed spores was associated with release of less than 0.2% of the total DPA present and no detectable loss in refractility. Thus loss of heat resistance is probably due to inactivation of part of the mechanism required to break the dormant state. This may be due to the creation of a heat-sensitive active enzyme by the action of L-alanine.

Boschwitz *et al.* (1983, 1985) found that NEM, PMSF, Tos-Lys- CH_2Cl and Tos-Arg-OMe all reversibly inhibited *Bacillus cereus* T spore germination at an early site. In the present study, all these compounds inhibited commitment, and PMSF and Tos-Arg-OMe also strongly inhibit loss of heat resistance. This is therefore evidence for the involvement of proteolytic activity in the commitment reaction. Procaine, which acts by membrane bulking and therefore disturbing the three-dimensional structure of the membrane (Lazdunski *et al.*, 1979), also reversibly inhibited the commitment event. Therefore it seems probable that part, or all, of the commitment process is associated with the inner (Racine *et al.*, 1981;

Rossignol & Vary, 1979) or outer membrane (Crafts-Lighty & Ellar, 1980).

From the results presented a putative germination pathway is proposed:

Heat shock activates the L-alanine receptor protein ($R \rightarrow R^*$), which, when triggered by L-alanine, becomes heat-sensitive ($R^* \rightarrow R^{**}$). This protein has been identified as being the first site of HgCl_2 inhibition (site I). R^{**} may have proteolytic activity which converts a proenzyme (L) into an active cortex-lytic enzyme (L^*), this enzyme also being HgCl_2 -sensitive (site II). Commitment is either a measure of the $R^* \rightarrow R^{**}$ or of the $L \rightarrow L^*$ reaction. Cortex hydrolysis then allows uptake of water and onset of all other downstream germination events, including loss of Zn^{2+} and DPA. In support of this model we have recently isolated an HgCl_2 -sensitive cortex-lytic enzyme activated early in the germination sequence; its properties await investigation.

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