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L-PROLINE SITE FOR TRIGGERING BACILLUS MEGATERIUM SPORE GERMINATION

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SUMMARY. Germination of Bacillus megaterium QM B1551 spores can be triggered by L-proline chloromethyl ketone at ~10 fold lower concentrations than L-proline. [^{3}H] L-proline chloromethyl ketone bound to several protein fractions, one of which was decreased in a mutant (JV137) that cannot be triggered by L-proline. Treatment of spores with [^{3}H] acetic anhydride specifically inhibited L-proline triggered germination, and also covalently modified the same protein fraction which appears to be bound to the spore membrane. These results indicate that it is possible to identify a protein fraction in spores that may play a key role in triggering spore germination.

INTRODUCTION. The biochemical mechanism by which a compound such as L-proline can trigger bacterial spore germination is not known. Previous work has suggested that the trigger mechanism may not require metabolism or the generation of a protonmotive force (1,2) and our present model (3) includes a unique receptor site for L-proline, possibly on the spore membrane. To probe the L-proline receptor site we have used an affinity analog of L-proline, L-proline chloromethyl ketone* (PCK), which may covalently bind to this receptor.

MATERIALS AND METHODS. B. megaterium QM B1551 spores were grown in supplemented nutrient broth, harvested, extracted with SDS-DTT and stored as previously described (4,5). Strain JV137 which cannot trigger germination on L-proline (1) was grown as above. All references to spore weights are on a dry weight basis.

Soluble cell fractions were obtained from SDS-DTT treated spores by lysozyme treatment (4) followed by sonic oscillation, 3-4 times for 30 s each, in the presence of glass beads (4). A supernatant fraction was obtained by centrifugation at 10,000 x g for 10 min. Samples were made up to 0.1 M Tris (pH 8), 2% SDS, 0.13 M DTT, and boiled for 2.0 min followed by chromatography on Biogel A1.5M (1.2 x 50 cm) in 1% SDS, 0.1 M Tris (pH 8) by the method of Fish et al. (6). [G-^{3}H]-L-proline (1 mCi/mmol) was used for the synthesis of N^{α}-tert-butoxycarbonyl-L-proline by the method of Nagasawa et al. (7). Synthesis of PCK was by a modified method (8) from N^{α}-tert-butoxycarbonyl-L-proline.

Spores (0.5 mg/ml) were acetylated by suspension in 10 mM sodium borate (pH 9.0), 0.1% (v/v) Ac_2O and the pH was maintained between 8.8 and 9.0 by the addition of 1 N NaOH at 25° (9). After 20 min, the spores were sedimented at 10,000 x g for 10 min, washed once, resuspended in H_2O and stored on ice. The same results were obtained using 0.05% Ac_2O and spores at 5 mg/ml and these

*Abbreviations: Ac_2O, acetic anhydride; DTT, dithiothreitol; PCK, L-proline chloromethyl ketone; SDS, sodium dodecyl sulfate; Tris, tris-(hydroxymethyl)-aminomethane-HCl.

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latter conditions were used with [^{3}H] Ac_2O to increase the quantity of acetylated spores while maintaining a high specific activity of [^{3}H] Ac_2O.

Triggering of germination was measured by loss in absorbance at 660 nm with heat-activated spores (10 min at 60°) as previously described (4) except that L-proline or PCK was used at indicated concentrations. A 60% decrease in absorbance represents >95% germination (1,4).

RESULTS AND DISCUSSION. When heat activated spores were incubated in 10 mM Tris (pH 8) plus PCK, germination was triggered as measured by the decrease in absorbance at 660 nm (Fig. 1). The degree of triggering (absorbance loss) was dependent on the concentration of PCK, and the concentration requirements were about 10 fold lower with PCK than with L-proline. Therefore, PCK interacts with spores and triggers germination, presumably by interacting with the proline trigger site. The following observations support this hypothesis.

First, the ability of PCK to trigger germination cannot be attributed to contamination by reagents used to synthesize PCK, because none of those reagents were effective trigger compounds (data not shown). Second, the higher affinity of spores for PCK vs L-proline (Fig. 1) indicates that triggering could not be due to proline contamination or conversion of PCK to L-proline. Third, PCK did not trigger germination in a mutant (JV137) that cannot be triggered by L-proline. Fourth, PCK did not trigger the germination of heat-activated spores which had been pretreated with Ac_2O, a chemical modification which inhibits L-proline triggered but not glucose triggered germination (10). Finally, ^{3}H-PCK was bound to spores during the first 2 min of triggering germination (data not shown), while radioactive proline binding was undetectable (1), therefore, PCK may be covalently bound to the proline receptor site.

In order to identify the products of PCK binding, heat-activated spores were incubated in 10 mM Tris (pH 8), 0.16 mM [^{3}H] PCK (1 mCi/mmol) for 15 min at 30°, and washed 4 times by centrifugation. The spores were disrupted and the soluble fraction analyzed by chromatography as described in Materials and Methods. As shown in Fig. 2, there were 4 major radioactive peaks. Peaks a and b but not c and d are protein, based on their insolubility in 5% TCA and sensitivity to proteinase K. Also peaks a and b but not c and d co-purified with spore membranes. We think that the material in peak b may contain the

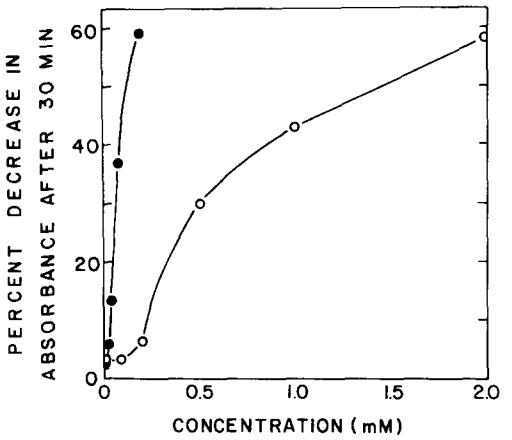

Fig. 1. Triggering of germination by PCK and L-proline. SDS-DTT treated spores were heat activated 10 min at 60° and diluted to 200 µg/ml in 10 mM Tris buffer (pH 8) plus PCK or L-proline at the indicated concentrations. The final percent loss in absorbance after 30 min in PCK (●——●) or L-proline (O——O) was plotted vs concentration of trigger compound. Similar results were obtained for spores at 1 mg/ml or with non-extracted spores.

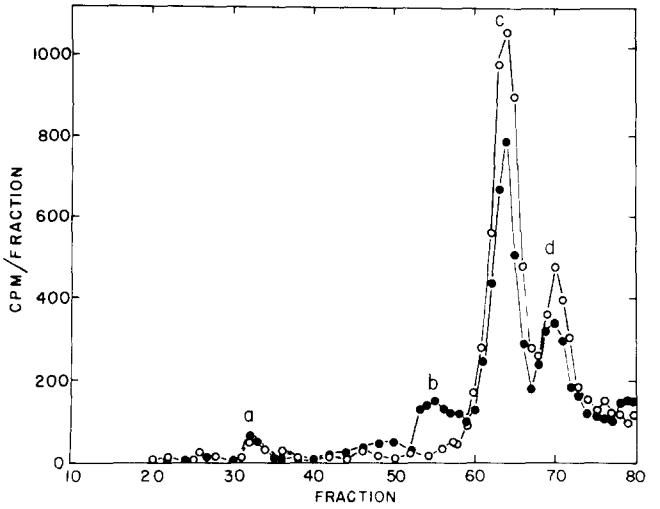

Fig. 2. Chromatography of radioactively labelled products. Spores of wild type (●——●) or JV137 (O——O) were incubated in 10 mM Tris (pH 8) with 0.16 mM [^{3}H] PCK (1 mCi/mmol) for 15 min at 30°, washed, disrupted, and the supernatant fraction was obtained and solubilized as described in Materials and Methods. A sample containing 1.39 mg protein was applied to a Biogel A1.5M column (1.2 x 50 cm) and eluted at 8 ml/hr with 0.1 M Tris (pH 8) 1% SDS. Fractions (0.7 ml) were collected, and 0.35 ml of each was mixed with 4 ml of a toluene based scintillation fluid (4) containing 33% Triton X-100 and counted in a scintillation spectrometer. The background radioactivity (30 cpm) was subtracted from each fraction. Also the radioactivity in the pellet obtained after centrifugation at 10,000 x g was analyzed by boiling in 2% SDS, 0.1 M DTT. After chromatography as above, no additional peaks of radioactivity were found.

proline trigger or recognition site based on the following data. First, when the mutant JV137 was labelled with [^{3}H] PCK as above, the radioactivity in peak b was diminished with respect to wild type (Fig. 2). Second, when spores were treated with [^{3}H] Ac_2O, disrupted and analyzed by chromatography as described above, only two major membrane associated peaks appeared which corresponded to the positions of peaks a and b in Fig. 2. The [^{3}H] Ac_2O radioactivity in peak a could be removed *in vitro* with 1 M hydroxylamine at pH 7, while the radioactivity could be removed from both peaks a and b at pH 9. This correlates well with the fact that if Ac_2O labelled spores, which do not trigger on L-proline (10), were treated with 1 M hydroxylamine at pH 7 there was no effect but at pH 9 the spores regained the ability to trigger germination on L-proline (Rossignol, D.P. and Vary, J.C., Abstracts of the XIth International Congress of Biochemistry).

These data suggest that the site of proline interaction in the spore may be identified and isolated using [^{3}H] PCK. These experiments are being pursued by analyzing the number of proteins in peak b of Fig. 2, isolation of revertants of strain JV137 and further analysis of the proteins labelled with Ac_2O. If the trigger site can be isolated in native form, its interactions with L-proline may be analyzed with biophysical techniques to study the trigger mechanism.

To our knowledge, these data are the first demonstration that a spore germination trigger site can be identified.

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