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PHOSPHOGLYCERATE MUTASE IN DEVELOPING FORESPORES OF BACILLUS MEGATERIUM MAY BE REGULATED BY THE INTRASPORAL LEVEL OF FREE MANGANOUS ION

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SUMMARY: When young intact forespores of Bacillus megaterium were incubated with either Mn++ or the ionophore X-537A, the pool of 3-phosphoglyceric acid (3-PGA) was stable. However, incubation of forespores with Mn++ plus the ionophore X-537A resulted in rapid and complete utilization of the 3-PGA. This effect was not seen with Ca++ or Mg++, and was also not observed with older forespores or fresh dormant spores. Since the phosphoglycerate mutase of B. megaterium has an absolute and specific requirement for Mn++, it is possible that phosphoglycerate mutase in developing forespores may be inactive because of a low intrasporal level of free Mn++.

INTRODUCTION: A large amount of 3-phosphoglyceric acid (3-PGA) is accumulated within the developing forespore late in the sporulation of Bacillus megaterium (1). This pool of 3-PGA is stable in both isolated forespores and dormant spores (1, 2), yet both developing forespores and dormant spores contain all the enzymes necessary for 3-PGA metabolism (2). Indeed, 3-PGA is completely metabolized in the first minutes of spore germination by dormant spore enzymes; the metabolism of this 3-PGA generates much of the ATP and NADH needed in the first minutes of spore germination (3,4). These findings indicate that there is a mechanism operating in the forespore to maintain some enzyme(s) of 3-PGA metabolism in an inactive form to allow for both the accumulation of 3-PGA in the developing forespore and the stability of the 3-PGA pool once accumulated. A mechanism often suggested for the metabolic dormancy of bacterial spores is their low water content, which would result in little or no enzyme action (5). However, estimates of the time in sporulation when forespore water content is

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drastically reduced indicate that this event occurs at the time of acquisition of spore heat resistance, about 1-2 hours after 3-PGA accumulation (2,5).

Previous work (1) as well as our own (Singh, R. P. and Setlow, P., unpublished results) has indicated that the phosphoglyceric acid pool in spores is > 99% 3-PGA with no detectable 2-PGA. This finding implicates phosphoglycerate mutase as one enzyme of 3-PGA metabolism which must be inactive in forespores. The phosphoglycerate mutase from cells of Bacillus species has the interesting property of not requiring 2,3-diphosphoglycerate for activity, but rather having an absolute and specific requirement for Mn++ (6, and Singh, R. P and Setlow, P., unpublished results). Consequently, w. attempted to alter the endogenous free Mn++ level in developing forespores by use of the ionophore X-537A which binds Mn++ and is able to transport this ion across a hydrophobic barrier as it does for Ca++ and Mg++ (7, and Singh, R. P. and Setlow, P., unpublished results). Our results suggest that changes in the Mn++ level within forespores can regulate the metabolism of the 3-PGA pool.

MATERIALS AND METHODS: The ionophore X-537A was the generous gift of Dr. W. E. Scott of Hoffman-LaRoche. The organism used in these studies was Bacillus megaterium QM B1551 originally obtained from Hillel Levinson (U.S. Army Labs, Natick, Mass.). Cells were grown at 30°C in supplemented nutrient both as previously described (8), and forespores were isolated from sporulating cells using the phosphate protoplast buffer as previously described (2). Forespores were then washed 2 times by centrifugation with cold Tris-protoplast buffer (0.6 M sucrose, 50 mM Tris-HCl (pH 7.4) and 16 mM $MgSO_4$), and suspended in warm (30°C) Tris-protoplast buffer at a forespore concentration two-fold higher than in the original culture. Samples were incubated at 30°C and aliquots (5 ml) were taken at various times, centrifuged (5 min, 10,000 xg) and the pellets frozen in an ethanol dry ice bath. Small molecules were extracted from these pellets with boiling 80% n-propanol as previously described (3).

Dipicolinic acid (DPA) was analyzed by the method of Rotman and Fields (9). 3-PGA was determined as ATP using the luciferase assay (3) after conversion of 3-PGA to ATP using ADP plus enolase, phosphoglycerate mutase and pyruvate kinase. Samples of forespore extract containing up to 10 nmoles of 3-PGA were incubated in a volume of 0.5 ml containing 50 mM Tris-HCl (pH 7.4), 100 mM KCl, 5 mM $MgSO_4$, 300 µM ADP, 20 µg of rabbit muscle pyruvate kinase (Sigma), 18 µg of homogeneous enolase from B. megaterium spores (10), and 25 µg of partially purified (to ∿ 25% purity) phosphoglycerate mutase from B. megaterium cells (Singh, R. P. and Setlow, P., unpublished results). The latter enzyme was free of adenylate kinase. After incubation for 5 min at 37°C the reaction mixes were boiled for 5 min and aliquots assayed for ATP. Controls were run to correct for ATP in the ADP (∿ 0.2%), ATP present in forespore extracts (< 10% of 3-PGA), to ensure that any 3-PGA present was indeed converted into ATP, and to ensure that levels of 2-PGA and phosphoenolpyruvate were low (< 5% of 3-PGA levels).

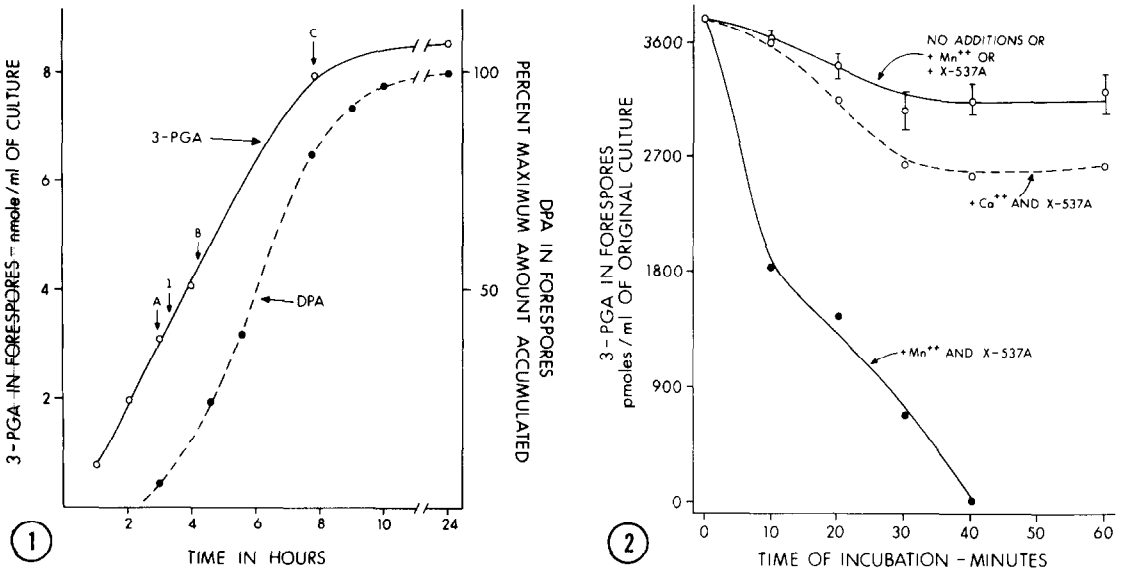

Fig. 1 - Forespore levels of DPA and 3-PGA during sporulation. Cells were sporulated and forespores isolated at various times and analyzed for DPA and 3-PGA as described in Materials and Methods.

Fig. 2 - Effect of the ionophore X-537A and various metal ions on forespore 3-PGA levels. Forespores were isolated at the point indicated by arrow 1 in Fig. 1, and incubated and analyzed as described in Materials and Methods. Additions to various incubations were: $CaCl_2$, 1 mM; $MnSO_4$, 1 mM; X-537A - 5 µg/ml. Since the X-537A was added from a 1 mg/ml solution in ethanol, all incubations also contained 0.5% ethanol. Note that Mg++ was present in all incubations at 16 mM to maintain forespore integrity.

RESULTS AND DISCUSSION: As has been previously found, 3-PGA was accumulated during sporulation about 1-1/2 hours before accumulation of DPA (2)(Fig. 1). When isolated forespores (taken at arrow 1, Fig. 1) were incubated at 30°C in Tris-protoplast buffer, there was only a slight (∿ 15%) loss in 3-PGA after 60 min (Fig. 2). Addition of either the ionophore X-537A or Mn++ gave no increase in the rate or magnitude of 3-PGA loss; similarly, addition of the ionophore plus Ca++ caused only a slightly greater loss in 3-PGA (Fig. 2). However, addition of both Mn++ and ionophore resulted in complete loss in 3-PGA by 40 min (Fig. 2). The loss in 3-PGA upon incubation with Mn++ and ionophore was not due to forespore lysis, since lysis was not observed in the phase contract

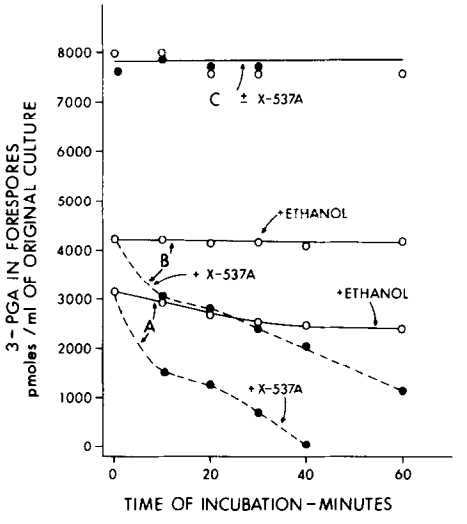

Fig. 3 - Effect of forespore age on the response of the 3-PGA depot to Mn++ plus X-537A. Forespores were isolated at the times indicated by the lettered arrows in Fig. 1, incubated as described in Materials and Methods with the addition of $MnSO_4$ (1 mM) and either ethanol (0.5%) or ethanol (0.5%) plus X-537A (5 µg/ml). Aliquots were taken extracted and analyzed for 3-PGA. The letters on different curves refer to the time of forespore harvest as denoted by the arrows in Fig. 1.

microscope. It was also not due to 3-PGA excretion, since no 3-PGA (< 5% of total) was found in the supernatant fluid from forespore samples (data not shown). Thus it seems possible that the combination of Mn++ and ionophore has stimulated the endogenous metabolism of the 3-PGA depot within the forespore.

The effect of the ionophore could only be obtained with young forespores (Fig. 3). Whereas forespores containing little 3-PGA (and much less DPA) lost their 3-PGA rapidly upon incubation with Mn++ plus ionophore, slightly older forespores gave a slower and less complete response (Fig. 3). Similarly, the oldest forespores tested, which contained the maximum amount of 3-PGA and ∿ 60% of the maximum amount of DPA, lost no 3-PGA on incubation with Mn++ plus ionophore (data not shown). Possibly, the layers of spore coat protein and spore cortex which have accumulated in the older forespores and in dormant spores prevented approach of the ionophore to some spore membrane (2).

Although there are many questions remaining concerning the experi-

ments described in this communication, the basic finding that Mn++ plus the ionophore X-537A caused an apparent reactivation of phosphoglycerate mutase within forespores of B. megaterium may have great significance. This finding suggests the possibility that the inactivity of phosphoglycerate mutase in forespores is due in part to a low level of free Mn++, an ion which is specifically required by this enzyme (6). Inactivation of this enzyme in forespores would largely block ATP production, since enzymes of the tricarboxylic acid cycle are not present in forespores (2). Thus, decreased free Mn++ levels may well play an important role in the onset of the metabolic dormancy of the bacterial spore. In light of this suggestion, several other points should be made. 1) Oh and Freese have shown that growth of B. subtilis in a Mn++ poor medium results in intracellular accumulation of 3-PGA (6). Thus, Mn++ deficiency can result in 3-PGA accumulation in vivo in Bacillus species. 2) Decreased endogenous levels of free Ca++ are thought to be involved in causing the dormancy of the unfertilized sea urchin egg (11). Dormancy in this system can be broken without fertilization by use of a Ca++ ionophore; similarly normal egg fertilization is accompanied by breaking of dormancy and a rise in endogenous free Ca++ levels (11). Possibly, imposition of metabolic dormancy by lowering endogenous divalent cation levels is a mechanism used not only in this latter system, but also in bacterial spores and possibly other dormant systems such as yeast and fungal spores as well.

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1. Nelson, D. L. and Kornberg, A. (1970) J. Biol. Chem. 245, 1137-1145
2. Singh, R. P., Setlow, B. and Setlow, P. (1977) J. Bacteriol. 130, 1130-1138
3. Setlow, P. and Kornberg, A. (1970) J. Biol. Chem. 245, 3637-3644
4. Setlow, B., Shay, L. K., Vary, J. C. and Setlow, P. (1977) J. Bacteriol. 132, 744-746
5. Gould, G. W. and Dring, G. j. (1975) Nature 258, 402-405
6. Oh, Y. K., andFreese, E. (1976) J. Bacteriol. 127, 739-746
7. Pressman, B. C. and deGuzman, N. T. (1975) Ann. N. Y. Acad. Sci. 264, 373-386
8. Setlow, P. and Kornberg, A. (1969) J. Bacteriol. 100, 1155-1160
9. Rotman, Y. and Fields, M. F. (1967 Anal. Biochem. 22, 168
10. Singh, R. P. and Setlow, P. (1978) J. Bacteriol. (in press)
11. Epel, D. (1977) Sci. Amer. 237, 128-131