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Regulation of phosphoglycerate mutase in developing forespores and dormant spores of *Bacillus megaterium* by the *in vivo* levels of phosphoglycerate mutase inhibitor

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Abstract. *Bacillus megaterium* accumulated 3-phosphoglycerate during sporulation which was utilized during spore germination. During sporulation a protein was synthesized before or at the start of 3-phosphoglycerate accumulation inside the developing spores about 1.5 h before dipicolinic acid accumulation. This protein has an affinity for Mn^{2+} and other divalent metal ions and inhibits phosphoglycerate mutase activity which has been shown to require Mn^{2+} However, the levels of the inhibitor decreased considerably (75-85%) during spore germination. No appreciable amount of the inhibitor was detected in the vegetable cell and mother cell compartment; however, the forespore compartment possesses an activity comparable to that of dormant spores. The partially purified inhibitor has a molecular weight of 11,000 and possesses both high and low affinity binding sites for Mn^{2+} and Ca^{2+} as determined by Scatchard plot analysis.

Keywords. *Bacillus megaterium;* 3-phosphoglyceric acid; phosphoglycerate mutase; mutase inhibitor; dipicolinic acid.

Introduction

Dormant spores of various Bacillus species contain an appreciable amount of 3-phosphoglyceric acid (3-PGA) which is accumulated late at a time in sporulation only within the developing forespores (Nelson and Kornberg, 1970; Setlow and Kornberg, 1970a, b; Singh *et al.,* 1977). The 3-PGA depot is stable in the forespore and dormant spore despite the presence of enzymes capable of catabolizing it, namely phosphoglycerate mutase (EC 2.7.5.3) enolase (EC 4.2.1.11) and pyruvate kinase (EC 2.7.1.40) at levels similar to those in growing cells and germinated spores (Singh *et al.,* 1977). Since 3-PGA accumulates in forespores even though the enzymes for 3-PGA catabolism are detected in extracts, at least one of these enzymes must have very little activity *in vivo* and become activated upon spore germination.

Abbreviations used: 3-PGA, 3-phosphoglyceric acid; 2-PGA; 2-phosphoglyceric acid; DPA, dipicolinic acid; SNB, supplemented nutrient broth; DEAE, diethylamino ethyl.

Previous work has implicated phosphoglycerate mutase as a key enzyme in the regulation of 3-PGA accumulation during sporulation (Singh and Setlow, 1978a, and 1979b). In this communication data are presented to show that phosphorglycerate mutase is the enzyme which is regulated to allow the accumulation of 3-PGA during sporulation followed by its utilization during spore germination. The regulation of this enzyme *in vivo* appears to be mediated, at least in part by the levels of free Mn^{2+}. The levels of free Mn^{2+} are likely to be controlled by the phosphoglycerate mutase inhibitor which is synthesised during sporulation and degraded upon spore germination, thereby relieving the inhibition of phosphorglycerate mutase.

Materials and methods

Chemicals and enzymes

Diethylaminoethyl—(DEAE)—cellulose and Sephadex G-100 were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. All other reagents were supplied by Sigma Chemical Co., St. Louis, Missouri, USA. Phosphoglycerate mutase and enolase were purified from log phase cells of *B. megaterium* as described by Singh and Setlow (1978b, 1979a). ADP was freed of contaminating ATP by incubating with hexokinase (EC 2.7.1.1) and glucose and subsequent boiling.

Organism, cultural conditions and isolation of forespores

All work described in this manuscript was carried out with *B. megaterium* QMB 1551, originally obtained from H.S. Levinson, U.S. Army Natick Laboratories, Natick, Massachusetts, USA. The organism was grown in a supplemented nutrient broth at 30°C, allowed to sporulate and were cleaned as described by Setlow and Kornberg (1969). The forespores were prepared as described earlier (Singh *et al.,* 1977).

Isolation and purification of phosphoglycerate mutase inhibitor

Lyophilized dormant spores were disrupted in dry state using Dental Amalgator (Wig-L-Bug) with acid cleaned sand as the abrasive (Sacks and Bailey, 1963). The dry powder was extracted for 1 h with ice-cold buffer A Tris (hydroxymethyl) aminomethane (Tris)–HCl, 50 mM; EDTA, 25 mM; pH 7.4 followed by centrifugation at 10,000 *g* for 20 min. The supernatant was discarded and the pellet washed thrice with Tris-HCl, pH 7.4, 50 mM. The residual material was dissolved in 50 mM Tris buffer pH 7.4 containing 5 mM $CaCl_2$ followed by incubation overnight at 37°C. The mixture was centrifuged and the pellet was reincubated as described above. Supernatants from both the preparations were pooled and dialysed overnight aganst Tris-buffer (50 mM) with three changes. The other steps followed in the purification of mutase inhibitor are listed in table 1. Mutase inhibitor was also isolated from forespores, germinated spores and the vegetative cells.

Assay of 3-PGA, phosphoglycerate mutase and mutase inhibitor

3-PGA was determined using the luciferase assay method (Setlow and Kornberg,

1970b) after conversion of 3-PGA to ATP using ADP plus enolase, phosphoglycerate mutase and pyruvate kinase. Samples of forespore or dormant spore extracts containing up to 10 n mol 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 rabit muscle pyruvate kinase, 18 µg of homogenous enolase prepared by the method of Singh and Setlow (1978) and 25 µg of purified phosphoglycerate mutase from *B. megaterium* prepared by the method of Singh and Setlow (1979a). The latter enzyme was free of adenylate kinase. After incubation of 5 min at 37°C, the reaction mixture was boiled for 5 min and an aliquot assayed for ATP. A control was run to correct for ATP contamination in ADP (<0.2%).

Assays of phosphoglycerate mutase utilized the discontinuous assay procedure described previously (Czok, 1974) where the first incubation containined only enzyme plus 3-PGA. After boiling, a second incubation was used to determine the amount of 2-PGA formed in the first incubation. The mutase inhibitor was assayed by adding in the first incubation mixture. The amount of mutase inhibitor which gives 50% inhibition of phosphoglycerate mutase is defined as one unit of inhibitor, units/mg of protein is called as specific activity.

Extraction of 3-PGA and DPA from foresp ores and dormant spores

Forespores were isolated at designated times (arrows in figure 1) as described earlier (Singh *et al.,* 1977) and spores prepared by growing *B. megaterium* in supplemented nutrient broth (SNB) medium. Spore preparation was free of vegetative cells and cell debris and contained 95% refractile forms when viewed in a phase contrast microscope (Setlow and Kornberg, 1969). 3-PGA and DPA were extracted from freeze dried forespores and dormant spores by boiling in 80% 1-propanol (Setlow and Kornberg, 1970a).

Analytical assays

DPA was assayed by the method of Rotman and Fields (1967). Protein was determined by following the procedure of Lowry *et al.* (1951). Growth of the organism was monitored by measuring absorbance at 600 nm with Spectronic-20 (Bausch and Lomb) spectrophotometer. Molecular weight of the mutase inhibitor was determined by methods published earlier (Whitaker, 1963; Weber and Osborne, 1969).

Binding efficiency of mutase inhibitor

Binding efficiency of the mutase inhibitor was determined by the equilibrium dialysis method (Englund *et al.,* 1969) except that the diameter and depth of the chambers was about 200 µl. The dialysis membrane used was Visking 20/32 type which had been boiled for 5 min in 5% Na_2CO_3 – 50 mM EDTA washed thrice with distilled water and stored in 50% ethanol at 4°C. Before use, membrane was hydrated in distilled water and blotted dry. To assemble the cells, approximately circular pieces of membrane about 0.5 inch diameter were placed between the two chambers in each set and the cells were then clamped into metal holders. Using

Hamilton syringe, 100 µl of mutase inhibitor, 25 mM Tris buffer pH 7.4 and 100 mM NaCl were placed in the chamber on one side of the membrane and 100 µl of distilled water, 25 mM Tris buffer pH 7.4, 100 mM NaCl and $MnCl_2$ (varying concentrations) containing 5 µl ^{54}Mn (5000 cpm) was placed on the other side of the membrane. The entire apparatus was shaken (25 rpm) at room temperature. After equilibration of the dialysis content (about 2 h), cell samples were removed from each side of the membrane with 10 µl Hamilton syringe for determination of radioactivity and binding efficiency of mutase inhibitor was calculated (Scatchard, 1949).

Calculation of binding data

Data were plotted according to the rearrangement of the equation for binding of the ligand (Mn^{2+}) to a mutase inhibitor as described by Setlow and Mansour (1972).

$$\frac{\bar{n}}{c} = \frac{1}{K_D}(\bar{n} - n)$$

where c is the free Mn^{2+} concentration and $\bar{n}$ is the average moles of Mn^{2+} bound/ unit of mutase inhibitor. Plots of $\bar{n}$/c vs $\bar{n}$, yield a straight line which extrapolates to the abscissa at n, the number of identical, independent binding sites on the mutase inhibitor. The dissociation constant (K_D) was obtained from the slope of the line. The amount of Mn^{2+} bound to the inhibitor was determined from the difference in radioactivity between the two sides of the dialysis chamber. The radioactivity on the side lacking the enzyme inhibitor was used to calculate the concentration of the free ligand (Mn^{2+}).

Results

Accumulation of 3-PGA and mutase inhibitor

The organism grown in SNB medium accumulated 3-PGA (figure 1). Appearance

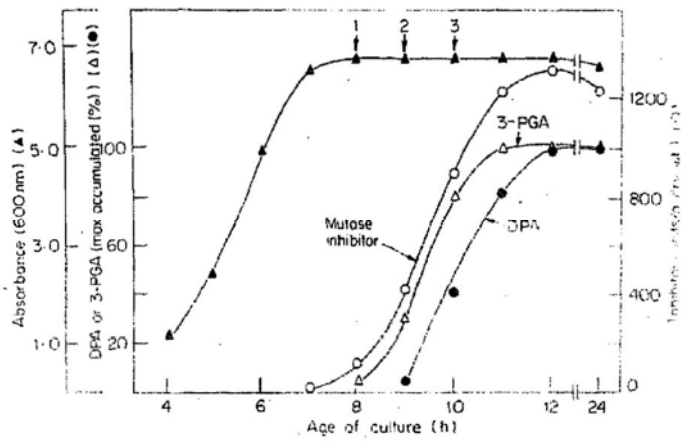

Figure 1. Accumulation of 3-phosphoglycerate acid, dipicolinate and mutase inhibitor during sporulation of *B. megaterium*. The organism was grown in supplemented nutrient broth and forespores were isolated at designated times as shown by arrows. 3-PGA, DPA and mutase were assayed.

of mutase inhibitor is quite interesting as it is likely to suppress the phosphoglycerate mutase activity *in vivo*. This possibly explained the accumulation of 3-PGA even in the presence of phosphoglycerate mutase, which was shown to require Mn^{2+} as a co factor. Accumulation of DPA seemed to play no significant role in regulating phosphoglycerate mutase activity in the developing forespores because it starts about 1.5 h after 3-PGA accumulation.

The levels of mutase inhibitor and 3-PGA vary with the stages of the growth (table 1). As expected no appreciable amount of mutase inhibitor and 3-PGA were detected in the vegetative cells and germinated spores under normal conditions. The levels of 3-PGA remained constant when spores were germinated in a medium containing NaF (Singh and Setlow, 1979b). Parallel observations have

Table 1. Levels of phosphoglycerate mutase inhibitor and 3-phosphoglyceric acid at different stages of development of *B. megaterium.*

Stage of growth	Inhibitor (Units/g dry wt.)	3-Phosphoglycerate (μmol/g dry wt.)
Dormant spores	1275	21.6
Germinated spores	69	1.2
Vegetative cells	Undetectable	4.2
Forespores[a]:		
Sample 1	155	11.5
Sample 2	490	17.2
Sample 3	900	20.1

[a] Forespores isolated from cultures withdrawn at the time designated by arrows in figure 1 and prepared according to the methods described in materials and methods section.

also been reported with the vegetative cells of *B. subtilis* when grown in a Mn^{2+} depleted medium (Oh and Freese, 1976).

Purification of mutase inhibitor

The mutase inhibitor was isolated from dormant spores and the purification steps followed are shown in table 2. DEAE-cellulose elution profile (figure 2) of mutase

Table 2. Purification of phosphoglycerate mutase inhibitor from dormant spores of *B. megaterium.*

Purification steps	Total units	Units/mg protein	Recovery (%)	Fold Purification
Spore extract	3644	10.5	100	1
Heat treatment	3500	197.0	96	18.8
DEAE cellulose	2032	960.2	55.7	91.4
Sephadex G-100	1019	1203.6	27	114.6

Phosphoglycerate mutase—inhibitor was assayed as mentioned in the materials and methods section.

inhibitor was obtained after eluting the column (2.5 × 25 cm) with 50 mM Tris buffer pH 7.4 (100 ml each) containing NaCl (0 to 0.5 M) in a linear gradient with a

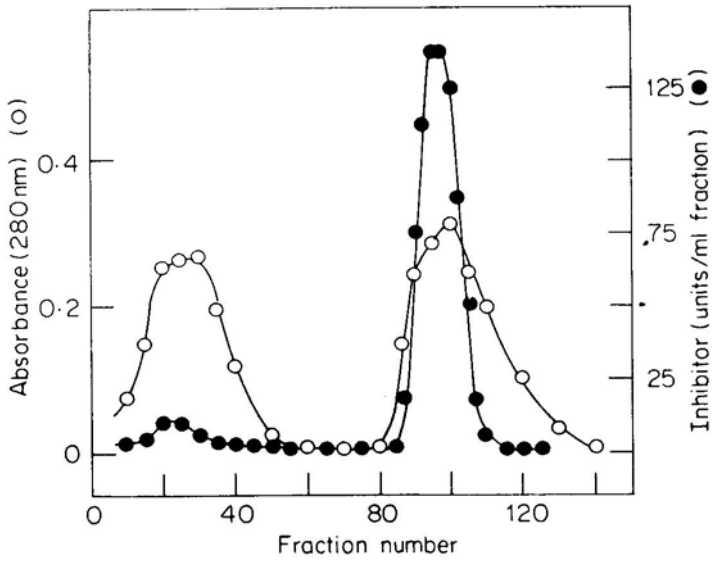

Figure 2. DEAE-cellulose chromatography of phophoglvcerate mutase inhibitor from dormant spores of *B. megaterium.* The spore extract (table 2, step 1) was dialysed overnight at 4°C against three changes of 50 mM Tris pH 7.4. The dialysed preparation was applied to a DEAE-cellulose column and eluted with a linear gradient from 0 to 0.5 M NaCl in 0.50 mM Trisbuffer pH 7.4).

flow rate of 25 ml/h. The fractions containing more than 50 units/ml were pooled and dialysed against 50 mM Tris buffer pH 7.4, concentrated using Pasteurpipette and dialysed again to remove salt. This concentrated preparation containing protein (50 mg) was layered on Sephadex G-100 column (2.5×72 cm) and eluted with Tris buffer (50 mM, pH 7.4) containing 100 mM NaCl with a flow rate of 5 ml/ h. This preparation was used for binding experiments and molecular weight determination.

Binding studies of mutase inhibitor

Binding properties of the mutase inhibitor with respect to Mn^{2+} and Ca^{2+} were done using the equilibrium dialysis technique. Results presented in table 3 show a

Table 3. Determination of binding constants of phosphoglycerate mutase inhibitor.

Addition	High affinity sites		Low affinity sites	
	K_D (μM)	n (nmol/ unit)	K_D (μM)	n (nmol/ unit)
$MnCl_2$ (3 mM)	0.35	0.29	47	1.82
$CaCl_2$ (2 mM)	1.2	0.47	60	2.32

dissociation constant of 47 μM for Mn^{2+}, suggesting that the binding of Mn^{2+} was not strong. Similar results were obtained when Ca^{2+} was substituted in place of Mn^{2+} (unpublished results). This suggestion was further strengthened with the exchange experiments using cold Mn^{2+} or Ca^{2+} as the case may be (unpublished data).

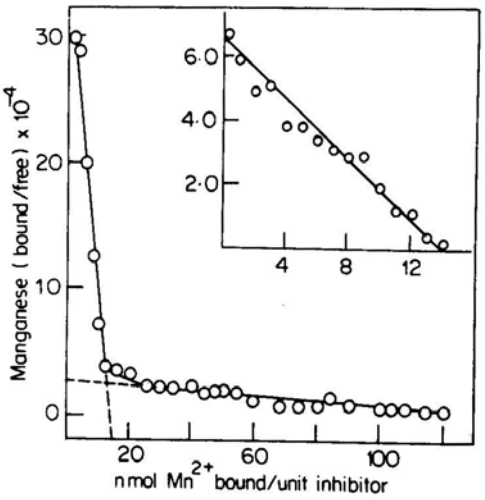

Figure 3. Manganese binding efficiency of mutase inhibitor. Binding studies were done by Scatchard plot analysis and binding constants, low and high affinity binding sites were calculated.

Scatchard plot analysis revealed the existence of low and high affinity binding sites which were determined by the extrapolation of lines on X and Y axis (figure 3). Table 3 shows the number of high and low affinity binding sites.

Discussion

3-Phosphoglycerate accumulated during the later stages of sporulation of *B. megaterium*.i.e. about 1.5 h before the accumulation of DPA (figure 1). Previously, Singh *et al.* (1977) have shown that most (>95%) of the 3-PGA was utilized during spore germination to produce ATP needed for other metabolic processes. It was also suggested that the enzymes of 3-PGA catabolism namely, phosphoglycerate mutase, enolase and pyruvate kinase are present in the various stages of growth with no detectable changes in specific activities when tested *in vitro*. Therefore, it is reasonable to presume that these enzymes act rapidly *in vivo* in the first minutes of spore germination. However, at least one of these enzymes must have little or no activity within the forespores or dormant spores in which a large stable pool of 3-PGA is accumulated. A reason which is often given for the absence of enzyme activity in dormant spores is their low water content (Gould, 1977; Gould and Dring, 1975). This may be true in the dormant spores, but is highly unlikely during 3-PGA accumulation in the forespores since 3-PGA accumulation is followed by other events like DPA accumulation which should require a hydrated spore core. Consequently, a different mechanism seems necessary to explain regulation of

enzymes of 3-PGA accumulation. Phosphoglycerate mutase has been shown to require Mn^{2+} for its activity (Watabe and Freese, 1979; Singh and Setlow, 1979b), and it could be activated inside the isolated forespores when incubated with Mn^{2+} plus ionophore (X 537A) (Singh and Setlow, 1979b). Interestingly, *B. megaterium* accumulated not only Ca^{2+} but also Mn^{2+} during sporulation, therefore, one can ask how phosphoglycerate mutase and its substrate (3-PGA) coexisted inside the forespore without interaction. A possible explanation was provided by Singh and Setlow (1979b) who proposed that in the developing spore the level of free Mn^{2+} was low resulting in the occurrence of inactive enzyme. It was further postulated that a metal binder might be synthesised during the sporulation which is degraded upon spore germination. This suggestion was examined and results showed that a mutase inhibitor accumulated inside the developing forespore. This accumulation coincides with the accumulation of a stable pool of 3-PGA but well before DPA synthesis. This mutase inhibitor is present only in forespores and dormant spores and no appreciable activity was detected either in vegetative cells or in the mother cell compartment and a significant decrease (>95%) in activity was noticed in germinated spores. Further the activity was lost when pretreated with chymotrypsin (unpublished data).

This inhibitor was isolated and purified from dormant spores and was found to be a protein with a molecular weight of 11,000. It binds Mn^{2+} preferentially over Ca^{2+} with a binding constant of $1.3\times10_5M^{-1}$ for Mn^{2+} and 1.7×10^5 M^{-1} for Ca^{2+}. The association constant for Mn^{2+} was in the order of 47 μM/unit of mutase inhibitor as determined by Scatchard plots analysis (Scatchard 1949). This analysis indicated the existence of high and low affinity binding sites on the mutase inhibitor which suggested that the mutase inhibitor tied up the free Mn^{2+} ions in the forespores at least where DPA concentration was less than 10% of the maximum as shown in figure 1 (arrows 1 and 2), because once DPA starts accumulating in the forespores it binds not only Mn^{2+} but also other divalent metals as DPA (Singh, 1982) is a known metal chelator. Since DPA begins to accumulate 1.5 h after 3 PGA accumulation the possibility of DPA inactivating phosphoglycerate mutase could be ruled out (Singh and Setlow, 1978a). From these data, it is still not possible to predict how mutase inhibitor selectively binds free Mn^{2+} inspite of the fact that more than 95% of Mn^{2+} from medium is taken up by the sporulating cells. It has already been demonstrated (Singh and Setlow, 1979b) that about 70% of the total Mn^{2+} is bound to DPA which is released upon spore germination along with DPA. Therefore, it is likely that the remaining Mn^{2+} is bound to mutase inhibitor at least partly ifnot completely. This fraction of the Mn^{2+} seems to be critical for phosphoglycerate mutase activity in forespores at least where the spore core is very much hydrated and could still accumulate stable 3-PGA pool.

It is difficult to suggest that the mutase inhibitor completely inhibits phosphor-glycerate mutase in the developing spores but it may contribute to its regulation although other factors like *in vivo* water content (Goud and Dring, 1975) and internal pH of forespores and spores (Setlow and Setlow, 1980) might also be

responsible in the inactivation of phosphoglycerate mutase activity. Further work is needed to locate the site of accumulation of the phosphoglycerate mutase inhibitor and to study the kinetics of inactivation of phosphoglycerate mutase by its inhibitor in the developing spores.

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References

Czok, R. (1974) *In Methods of enzymatic analysis* (Academic Press Inc., New York), Vol. 3, p. 1414.
Englund, P. T., Huberman, J. A., Jovin, T. M. and Kornberg, A. (1969) *J. Biol Chem.,* **244,** 3038.
Gould, G. W. (1977) *J. Appl Bacteriol.,* **42,** 297.
Gould, G. W. and Dring, G. J. (1975) *Nature (London),* **258,** 402.
Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol Chem.,* **193,** 625.
Nelson, D. L. and Kornberg, A. (1970) *J. Biol Chem.,* **245,** 1137.
Oh, Y. K. and Freese, E. (1976) *J. Bacteriol.,* **127,** 739.
Rotman, Y. and Field, M. I. (1967) *Anal. Biochem.,* **22,**168.
Sacks, L. E. and Bailey, G. E. (1963) *J. Bacteriol.*, **85,** 720.
Scatchard, G. (1949) *Ann. New York Acad. Sci.*, **51,** 660.
Setlow, P. and Kornberg, A. (1969) *J. Bacteriol.*, **100,** 1155.
Setlow, P. and Kornberg, A. (1970a) *J. Biol Chem.,* **245,** 3637.
Setlow, P. and Kornberg, A. (1970b) *J. Biol Chem.,* **245,** 3645.
Setlow, B. and Mansour, T. E. (1972) *Biochemistry,* **11,** 1478.
Setlow, B. and Setlow, P. (1980) *Proc. Natl Acad. Sci. (USA),* **77,** 2474.
Singh, R. P. (1982) *Indian J. Exp. Biol.,* **20,** 223.
Singh, R. P., Setlow, B. and Setlow, P. (1977) *J. Bacteriol.,* **130,** 1130.
Singh, R. P., and Setlow, P. (1978a) *Biochem. Biophys. Res. Commun.,* **82,** 1.
Singh, R. P. and Setlow, P. (1978b) *J. Bacteriol.,* **134,** 353.
Singh, R. P. and Setlow, P. (1979a) *J. Bacteriol.,* **137,**1024.
Singh, R. P. and Setlow, P. (1979b) *J. Bacteriol.,* **139,** 889.
Watabe, K. and Freese, E. (1979) *J. Bacteriol.,* **137,** 773.
Weber, K. and Osborne, M. (1969) *J. Biol. Chem.,* **244,** 4406.
Whitaker, J. R. (1963) *Anal. Chem.,* **35,** 1950.