Time Course of Purine Nucleoside Phosphorylase Occurrence in Sporulation of *Bacillus cereus*

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Purine nucleoside phosphorylase (EC 2.4.2.1) from *Bacillus cereus* T was examined at hourly intervals during growth and sporulation. The enzyme has maximal activity in extracts prepared from cells during stages I and II. The activity during exponential growth is only 6.6% of the maximum and that in free spores is only 3.3%. Conservation of the purine nucleoside phosphorylase during sporulation is apparent as shown by the gradual increase in heat resistance.

Purine nucleoside phosphorylase (EC 2.4.2.1) has previously been shown to be present in both spores and vegetative cells of *Bacillus cereus*. Lawrence (9) showed that inosine was cleaved by spores of *B. cereus* and would replace adenosine in the initiation of germination. The enzyme itself was originally identified in spores by Krask and Fulk (8). It was their belief that this enzyme participated in germination. Gardner and Kornberg (2) have shown that the synthesis of both the vegetative and spore forms of the enzyme are directed by the same cistron. Engelbrecht and Sadoff (1) previously showed that the half-life of the spore purine nucleoside phosphorylase is seven times that of the vegetative cell purine nucleoside phosphorylase at 60 C. However, at that temperature, the half-life of the spore enzyme is the same as that of the vegetative form in the presence of the substrate orthophosphate (1).

This report shows that purine nucleoside phosphorylase occurs at maximal activities in the early stages of sporulation. The changes in heat resistance of the enzyme in the course of sporulation will also be described.

MATERIALS AND METHODS

Inosine was obtained from Calbiochem, Los Angeles, Calif. Tris(hydroxymethyl)aminomethane (Tris) and streptomycin sulfate were purchased from Nutritional Biochemicals Corp., Cleveland, Ohio, and xanthine oxidase from Worthington Biochemical Corp., Freehold, N.J.

Bacillus cereus T originally obtained from the University of Illinois, Urbana, was used in this work.

The cells were grown at 30 C in a modified G medium of Hashimoto, Black, and Gerhardt (4) in a 15-liter New Brunswick Microferm fermenter employing an inoculum of 400 ml of exponentially growing cells. Entire cultures were harvested at various times up to 24 hr, by which time sporulation was complete. The cells were collected by centrifugation and frozen as a paste until used. Samples were taken for the determination of pH, turbidity, dry weight, and dipicolinic acid (DPA). Yields averaged 75 grams (wet weight) per 15 liters of medium.

Preparation of extracts and purine nucleoside phosphorylase purification. The frozen cells were broken in an Omnimixer (Sorvall) with an equal volume of glass beads (no. 110 pavement marking beads, Minnesota Mining and Manufacturing) in 0.05 M Tris-hydrochloride buffer, pH 7.5. Unbroken cells and debris were removed from extracts by centrifugation, and the specific activity of the phosphorylase was determined. The protein concentration was assayed spectrophotometrically (14) or by the method of Lowry et al. (10).

The extracts were treated with 1 ml of 5% streptomycin sulfate per 5 ml of extract to precipitate nucleic acids and facilitate ammonium sulfate fractionation. The procedure was carried out at 4 C overnight, followed by centrifugation. At ammonium sulfate precipitation the fraction precipitating at 40 to 80% saturation contained all the active enzyme. This fraction was suspended in a minimal amount of 0.05 M Tris-hydrochloride buffer, pH 7.5, and dialyzed overnight against the same buffer. Gel filtration through a G-200 Sephadex column (Pharmacia) yielded preparations purified 100-fold but of approximately 2% the purity of previously reported highly purified preparations (3).

Assay of enzyme. The modified spectrophotometric method of Kalckar (6) was used to assay for the presence of purine nucleoside phosphorylase.

One unit of enzyme was defined as that amount catalyzing the hydrolysis at 37 C of 1 μmole of inosine per min, corresponding to the International Unit (U). For convenience, the milliunit (mU) was used in defining specific activity.

The assay for DPA was performed according to the method of Janssen et al. (5).

RESULTS

The purine nucleoside phosphorylase activity in extracts varied with the age of the culture. Figure 1 shows the specific activity of the purine nucleoside phosphorylase relative to other parameters previously defined for the stages of sporulation; phase microscopy, optical density, DPA content, and pH of the culture. During exponential growth, the enzyme was present to the extent of 40 mU/mg of protein. At 12 hr, or early in sporulation, the level had reached 310 mU/mg. The level of enzyme reached a maximum (600 mU) at 13 hr (stage I) (11, 14) which was the time of axial filament formation. By the time engulfment of the forespore occurred and refractile bodies could be seen (stage III) (11, 14), the specific activity had dropped to the level found in the free spore, 20 mU/mg.

Thermal inactivation of the purine nucleoside phosphorylase occurs as a first order reaction at 50 C. The inactivation pattern is bimodal early in sporulation, the enzyme becomes progressively more stable, and reaches the maximal thermal stability in mature spores. At the time of its highest specific activity (12 to 15 hr) 50% of the enzyme has an intermediate stability with a half-life of approximately 40 min. The labile portion has a half-life of 15 min (Fig. 2) and a slope similar to that of the enzyme isolated from vegetative cells (1). By 16 hr, or at sporulation stage II (11, 14), all of the enzyme is of the intermediate thermal stability. The enzyme extracted from free spores (stage VII) at 24 hr has a half-life of 640 min. (Fig. 2).

DISCUSSION

A derepression of purine nucleoside phosphorylase synthesis occurs at the onset of sporulation resulting in high concentrations of the enzyme early in the process, suggesting an important role at this time. It has been shown previously that the equilibrium of the purine nucleoside phosphorylase reaction favors riboside formation (6), although it cleaves inosine, deoxyinosine, guanosine, deoxyguanosine, and 6-mercaptopurine ribonucleoside (2). Thus, the enzyme may participate in a purine salvage pathway and be involved in the extensive ribonucleic acid turnover which occurs during sporulation (7). The enzyme is present in small amounts in the exponentially growing vegetative cells and in the free spore. The activity level in the vegetative cell is 6.6% of the maximum observed and only one-half, or 3.3%, this quantity in the free spore. The functional role of the enzyme may be minimal at these time periods.
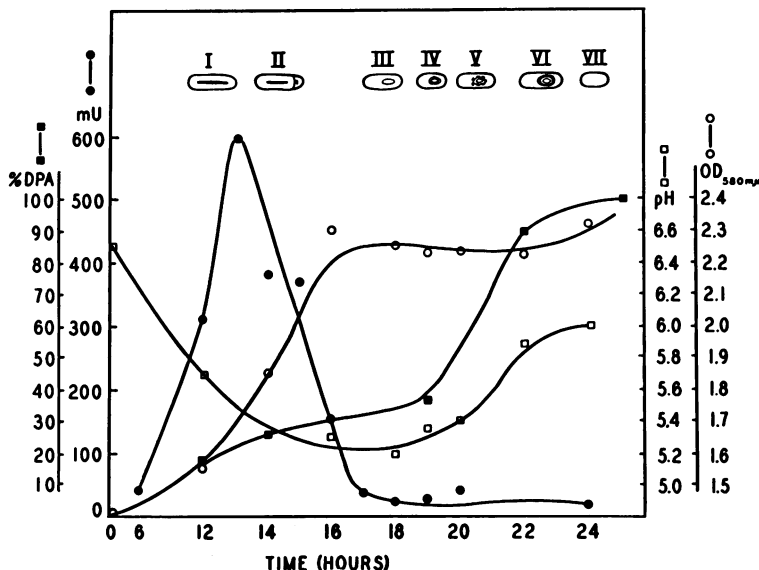

FIG. 1. *Specific activity of purine nucleoside phosphorylase (mU/mg of cell protein) from Bacillus cereus as related to optical density, pH of culture, dipicolinic acid (DPA) content, and microscopy.*
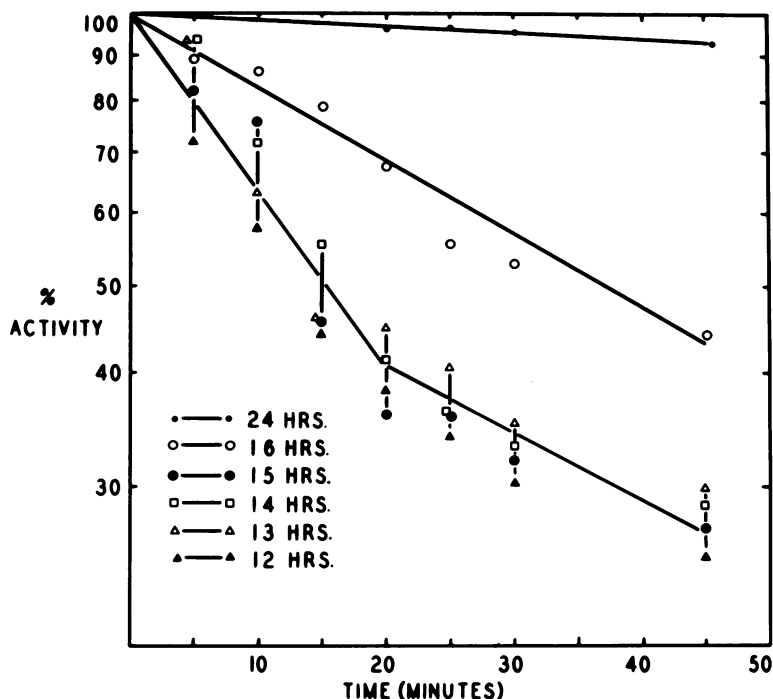
FIG. 2. *Thermal inactivation of purine nucleoside phosphorylase at 50 C. Enzyme samples in 0.05 M Tris-hydrochloride, pH 7.5, containing four units of phosphorylase per ml were heated for periods up to 45 min and a 0.4 unit was used for each spectrophotometric assay.*

A primary characteristic of spore formation is the development of heat resistance. This property must be acquired by all labile molecules which are incorporated into spores, as is the case of the purine nucleoside phosphorylase. The enzyme becomes more stable during sporulation in a process which seems to occur in a stepwise fashion. The bimodal inactivation curves suggest that at some stages part of the phosphorylase is labile, whereas another part appears as a stability intermediate between the vegetative and spore protein. The unstable enzyme is apparently segregated out of the spore so that, by the time the spore is free of the mother cell, only the stable form remains.

The phosphorylase does not appear to be cleaved by sporulation-specific protease (H. Engelbrecht, Ph.D. thesis, Michigan State Univ., East Lansing, 1968) as is the fructose, 1,6-diphosphate aldolase (12). However, a reduction in apparent enzyme molecular weight during sporulation has been shown to occur (1, 3). Gilpin and Sadoff (3) showed that the spore purine nucleoside phosphorylase in the absence of orthophosphate contained only two sodium dodecyl sulfate-dissociable polypeptide chains, whereas the vegetative-cell enzyme had four under the same conditions. How the modification of the enzyme occurs remains unknown.

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