THERMAL STIMULATION OF BACILLUS MEGATERIUM GLUCOSE-6-PHOSPHATE DEHYDROGENASE

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Received November 5, 1969

SUMMARY

The activity of glucose-6-phosphate dehydrogenase, extracted from vegetative cells of Bacillus megaterium, was increased 2-3 fold by heating at 25 C prior to assay. The stimulation, dependent on the ionic concentration and pH, was reversed by chilling to 0 C. The thermally stimulated enzyme contained 2 enzymatically active species, one of which migrated in a centrifugal field ahead of the single species of the non-stimulated enzyme.

In assaying Bacillus megaterium glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate:NADP oxidoreductase, E. C. 1.1.1.49) in crude extracts of spores or of vegetative cells derived from spores after 1 hr in microcycle sporogenesis (2), we noted that the initial reaction rates depended on the prior thermal history of the extract. Crude extracts, held at 0 C until dilution into the assay mixture were one-half as active as extracts which had first been warmed for 10 min at the assay temperature (25 C). This thermal stimulation was studied further, using a preparation of the vegetative cell enzyme, purified as described below.

Bacillus megaterium QM B1551 spores, produced on the modified medium, omitting agar, of Arret and Kirshbaum (1), were inoculated into 1-liter flasks containing 125 ml of Brain Heart Infusion (Difco), supplemented to give a final glucose concentration of 0.3%, and incubated, with shaking, for 6 hr at 30 C. The resulting vegetative cells were washed 8 times with 0.05M tris (hydroxymethyl) aminomethane ("Tris") buffer, pH 7.4, centrifuged, and broken at 0 C in a mixture of the same buffer and No. 13 Ballotini beads (pellet: buffer: beads: = 1 ml: 5 ml: 5 g), by 10-min treatment in a Minimill (Gifford-Wood Co., Hudson, N. Y.). The cell homogenate was centrifuged

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at 30,000 x g for 30 min and the supernatant fluid (crude extract) was dialyzed for a total of 2 hr at 0 C against 3 changes of the above buffer. Nucleic acids were precipitated by rapid addition of streptomycin sulfate (1.5%, w/v). The mixture was stirred for 10 min at 0 C, and centrifuged at 30,000 x g for 20 min. The supernatant fluid was dialyzed at 0 C for a total of 2 hr against 3 changes of a solution containing 300 g of $(NH_4)_2SO_4$ per liter of Tris buffer. The preparation was centrifuged as above, and the supernatant fluid was dialyzed against 3 changes of a solution containing 450 g of $(NH_4)_2SO_4$ per liter of Tris buffer. The precipitated protein, separated by centrifugation, was dissolved in, and dialyzed against the Tris buffer. The enzyme preparation, which remained in the supernatant after centrifuging at 100,000 x g for 1 hr and which had ca. 15-fold the specific activity of the crude extract, was used in the following studies.

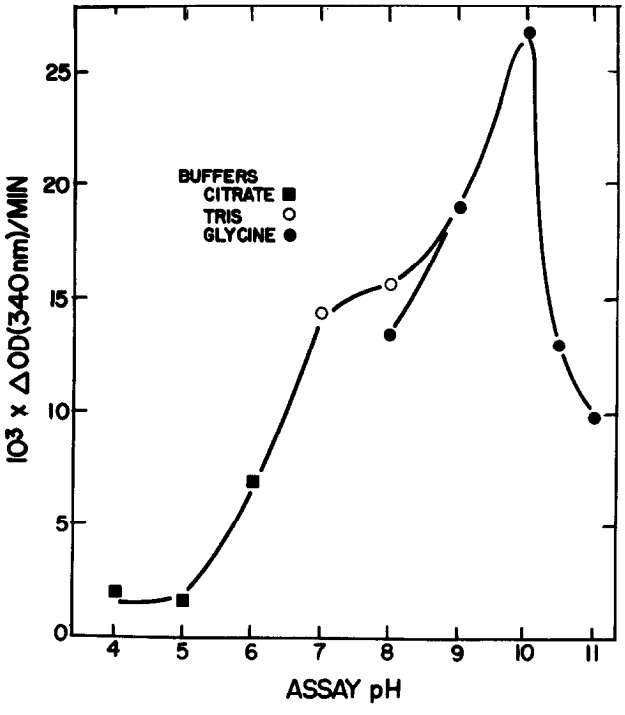

Figure 1. Effect of pH on Bacillus megaterium glucose-6-phosphate dehydrogenase. Enzyme, thermally stimulated by incubation at 30 C for 5 min in 0.05M sodium citrate, pH 5, was assayed (0.033M buffers) at pH 4 to 6 (sodium citrate buffer); at pH 7 and 8 (Tris buffer); or at pH 8 to 11 (glycine-KOH buffer).

Assays were routinely conducted in a total volume of 3 ml, containing 100 μmols of glycine-KOH buffer, pH 10; 50 μmols of $MgCl_2$; 10 μmols of glucose-6-phosphate; 200 μg (0.25 μmols) of NADP; and 0.01 to 0.05 ml of enzyme. NADP reduction was followed at 340 nm in a temperature-controlled (25 C) Beckman model DU spectrophotometer.

The enzyme displayed a peak of activity at pH 10 in glycine-KOH buffer (Fig. 1) and was half-maximally active in this buffer at pH 8 and at pH 10.5. The enzyme was NADP-specific, and was markedly stimulated by (but not absolutely dependent upon) Mg^{++} or Mn^{++}. Unlike rat mammary glucose-6-phosphate dehydrogenase (3), this bacterial enzyme was not inhibited by ATP, inorganic phosphate, or Mg^{++}.

Warming of the buffered enzyme at 25 C prior to its addition to the assay mixture resulted in a 2-3 fold increase in activity. A 10-min warming

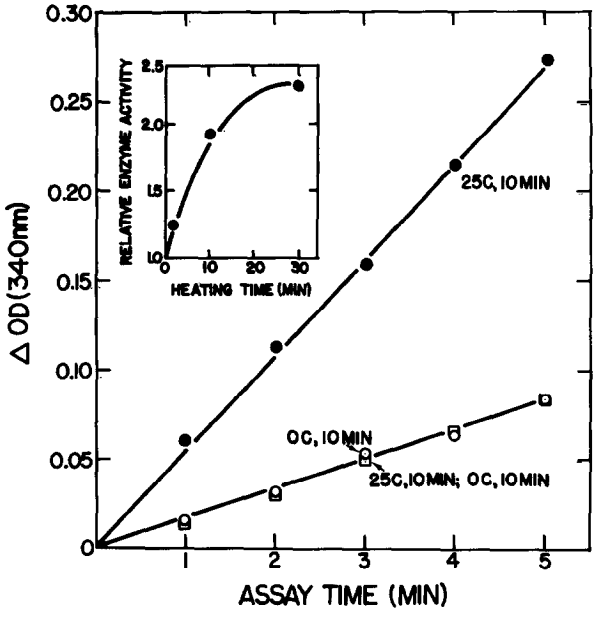

Figure 2. Thermal stimulation of Bacillus megaterium glucose-6-phosphate dehydrogenase and reversal by chilling. Before addition to the assay mixture, enzyme, at pH 10 (0.5M glycine-KOH buffer), was incubated either for 10 min at 0 C; for 10 min at 25 C; or for 10 min at 25 C followed by 10 min at 0 C. Insert shows effect of preassay heating time on stimulation of the enzyme (relative activity of enzyme preincubated at 0 C = 1.0).

at pH 10 (0.5M glycine-KOH buffer) was sufficient to yield a near-maximal response; a 30-min exposure produced little further stimulation (Fig. 2, insert). The thermal stimulation of the enzyme was reversed by a 10-min exposure to 0 C after the initial warming (Fig. 2), but was not reversed at temperatures between 20 and 30 C. The kinetics of the assay (Fig. 2) indicated enzyme stability during the assay, the process of thermal stimulation being stopped as a result of the dilution (1:60) in the assay mixture. Other experiments confirmed that neither thermal stimulation nor its reversal by chilling occurred with very dilute enzyme solutions. The enzyme was thermally stimulated (reversible by chilling) over a broad range of pH, provided that an appropriate ionic concentration was selected for each pH. For example, thermal stimulation (25 C) at pH 10 required 0.5M buffer (glycine-KOH), but there was no stimulation at this pH in 0.05M buffer; at pH 5, 0.05M buffer (sodium citrate) was required to provide equivalent stimulation (2-3 fold), but there was no increase in activity when the enzyme was warmed at this pH in 0.5M buffer.

We assumed that the most active form of the enzyme was in equilibrium with a less active form in a manner resembling the dimer: monomer equilibrium described by Levy et al. (3), and that the equilibrium constant was a function of temperature, ionic concentration, and pH. Confirmation of this assumption was sought in an ultracentrifugal separation of the 2 forms of the enzyme. Enzyme, either maintained at 0 C, or thermally stimulated at 25 C for 10 min at pH 10 (0.5M glycine-KOH) or at pH 5 (0.05M sodium citrate), was layered on Tris-buffered (pH 7.4) sucrose gradients (5-20% sucrose). The gradients were centrifuged (100,000 x g) for 20 hr at either 20 C (activating conditions) or at 0 C (nonactivating conditions). Fifty 0.11-ml aliquots, collected by dripping from the bottom of each centrifuge tube, were assayed for enzyme activity. The nonstimulated enzyme migrated in the centrifugal field (0 C) as a single species; centrifugation (at 20 C) of the thermally stimulated enzyme revealed, in addition, an enzymatically active species which migrated ahead of the original one. Similar results were obtained for enzyme stimulated at pH 5 and at pH 10 (Fig. 3).

On the bases of failure of thermal stimulation of very dilute solu-

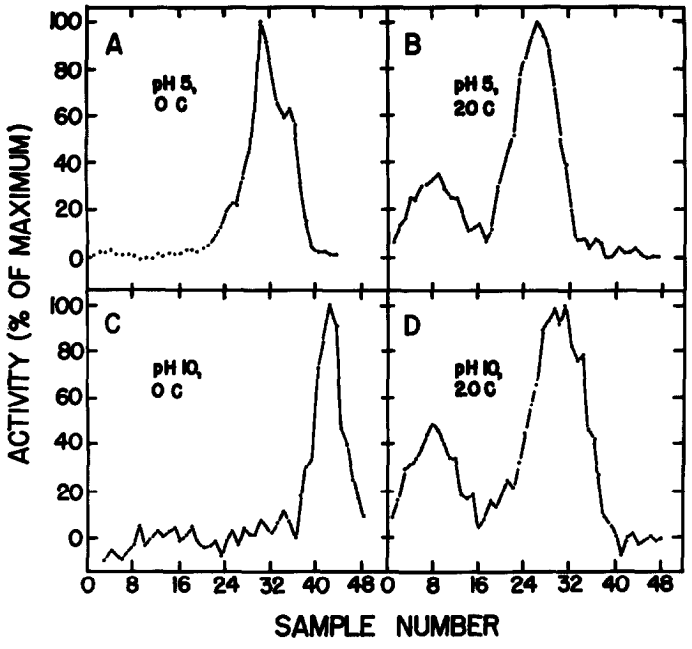

Figure 3. Centrifugal separation of nonstimulated and thermally stimulated forms of glucose-6-phosphate dehydrogenase from Bacillus megaterium. Nonstimulated enzyme and enzyme, thermally stimulated (25 C, 10 min) at pH 5 or 10, were layered on Tris-buffered (pH 7.4) sucrose gradients (5-20% sucrose) and centrifuged at 100,000 x g for 20 hr. Nonstimulated enzyme (A and C) was centrifuged at 0 C; thermally stimulated enzyme (B and D) was centrifuged at 20 C. Samples (0.11 ml) were collected (sample No. 1 was at bottom of centrifuge tube) and were assayed for 30 min. The noncoincidence in the position of the major peaks may be attributable to changes in protein partial specific volume with temperature; to a density change in solvent with temperature; or to slight changes in protein configuration with pH.

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tions of enzyme and of the ultracentrifugation data, we postulate that thermal activation of glucose-6-phosphate dehydrogenase involves a reversible conjugation of enzyme molecules to produce a larger and more active molecular species.

ACKNOWLEDGMENTS

We thank M. T. Hyatt and G. R. Mandels for their critical reviews of the manuscript.

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