Glycerol Protection and Purification of *Bacillus subtilis* Glucose Dehydrogenase*

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*Bacillus subtilis* glucose dehydrogenase (EC 1.1.1.47) has been purified from sporulating cell extract to apparent homogeneity (as determined by polyacrylamide gel electrophoresis, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and isoelectric focusing). The enzyme purified as a single molecular species with no evidence for a multiple form of the enzyme. The *B. subtilis* glucose dehydrogenase has an apparent isoelectric point of 4.7–4.8 and an apparent *M*ₐ = 126,000 and is comprised of four subunits of *M*ᵣ = 31,500 each. The glucose 2-deoxyglucose and glucosamine substrate specificity of the enzyme is similar to the substrate specificity for *B. subtilis* spore germination, suggesting that the spore glucose dehydrogenase may play some role in spore germination. The *B. subtilis* glucose dehydrogenase is extremely dependent on the presence of glycerol or other hydrophobic bond-stabilizing agents (or NAD) for retention of enzymatic activity, and the presence of glycerol (20% w/v) in the extraction and purification buffers was absolutely necessary for the successful purification of this enzyme.

Spore-forming bacteria such as *Bacillus subtilis* synthesize a number of new proteins during the formation of their heat-resistant endospores (1–7). One such protein is the enzyme glucose dehydrogenase (EC 1.1.1.47) (8, 9) which presumably functions during glucose-induced spore germination following preincubation of the spores with fructose, asparagine, and potassium (10, 11). Although the appearance of glucose dehydrogenase has been repeatedly used as a biochemical marker in the study of bacterial sporulation (12–18), the lability of *B. subtilis* glucose dehydrogenase (19) has been an obstacle in the purification, characterization, and use of this enzyme in biochemical (20) and genetic (21) studies of bacterial sporulation (22).

The present paper reports the glycerol stabilization of *B. subtilis* glucose dehydrogenase, thus permitting purification of the enzyme to apparent homogeneity, and reports some of the properties of the purified enzyme. It also reports the similarities between the carbohydrate substrate specificity for the purified enzyme and that for *B. subtilis* spore germination, suggesting that glucose dehydrogenase may play some role in glucose-initiated spore germination.

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**EXPERIMENTAL PROCEDURES**

**RESULTS**

Purification of the *B. subtilis* Glucose Dehydrogenase—Glucose dehydrogenase can be purified from *B. subtilis* spores (23). However, rather drastic means (use of glass beads and grinding in a cell mill) are needed to disrupt the spores. The enzyme can also be extracted from sporulating cells of *B. subtilis* by lysozyme sonication or French cell rupture (23). However, once the forespore has further matured into a completed spore, spore enzymes can no longer be obtained by these milder extraction procedures (13–14). To obtain sufficient quantities of the glucose dehydrogenase under mild extraction conditions, we have used a *B. subtilis* mutant (61297) blocked at sporulation stage V (24–26), i.e. after glucose dehydrogenase had been formed (17) but before cells have become resistant to milder disruption.

*B. subtilis* 61297 cells were grown in 189- and 300-liter batches in Yeast Extract-Tryptone Casenholtz salts medium (27) or Nutrient Sporulation medium (23) (see "Experimental Procedures"). The maximum level of glucose dehydrogenase was obtained from cells harvested about 5 h after the end of truly exponential growth (about 3 h after the culture turbidity was no longer increasing). At this time, about 10–20% of the cells showed phase bright sporangia with dark centers (normal *B. subtilis* sporangia at this same time would be completely phase bright); the appearance of these sporangia provided the indication of the culture's readiness for harvesting.

The sporulating cells were disrupted and the glucose dehydrogenase was purified as described under "Experimental Procedures." The purification procedure employed sequential column chromatography on DEAE-Sephacel, Ultragel ACA-34, hydroxyapatite, phenyl-Sepharose, and finally purification by chromatofocusing. The results of the individual purification steps are summarized in Table I. The presence of 20% (w/v) glycerol in all buffers was absolutely necessary for preservation of enzyme activity. The purified enzyme has been stored at −20 °C in 20% (w/v) glycerol, 0.05 M imidazole buffer (pH 6.5) for more than 4 years without any appreciable loss of activity. The necessity for a serine protease inhibitor phenylmethylsulfonyl fluoride and 2-mercaptoethanol in the...
buffers has not been directly demonstrated but these compounds were added as a precautionary measure. Phenylmethylsulfonyl fluoride was always added directly to the buffers in the dry form, or in ethanol, since aqueous solutions of phenylmethylsulfonyl fluoride rapidly lose their activity (28).

Properties of B. subtilis Glucose Dehydrogenase—The purified glucose dehydrogenase migrated as a single protein band in polyacrylamide gel electrophoresis (which co-migrated with glucose dehydrogenase activity as measured by the tetrazolium-linked assay) at pH values of 6.43 (Fig. S-8), 7.5, and 8.5. 20% (w/v) glycerol was added to the gel to preserve enzymatic activity and to prevent subunit dissociation which gives the appearance of multiple enzyme bands (29-31). Dissociation of glucose dehydrogenase into subunits followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed a single molecular weight species of $M_r = 31,500$ (Fig. S-9). Use of larger amounts of glucose dehydrogenase did not show the presence of any other protein bands (Fig. S-10).

Sepharose S-300 gel filtration of the purified B. subtilis glucose dehydrogenase showed a single molecular species with an apparent $M_r = 126,000$ (Fig. S-11), which is consistent with the enzyme having four similar molecular weight subunits. The fact that the value obtained by gel filtration is so close to the times the value obtained by sodium dodecyl sulfate-polyacrylamide gel electrophoresis subunit indicates that, unlike the B. subtilis inositol dehydrogenase (32), the B. subtilis glucose dehydrogenase is more globular in shape. (Sucrose density gradient centrifugation of the B. subtilis enzyme also indicated $M_r = 125,000$.)

The isoelectric point of the B. subtilis glucose dehydrogenase is 4.7-4.8 as determined by chromatofocusing (Fig. S-6) or by column isoelectric focusing (Fig. S-7). It is slightly more basic than the isoelectric point of the B. subtilis inositol dehydrogenase which is 4.4 (32).

**Glycerol Protection of B. subtilis Glucose Dehydrogenase**—The effect of buffer composition and the necessity of glycerol for the protection of B. subtilis glucose dehydrogenase activity were examined by dialyzing dilute quantities of both partially purified and purified enzyme against various buffers, pH values, and glycerol concentrations and determining the amount of glucose dehydrogenase activity remaining. Fig. 1 shows the effect of glycerol concentration on the stability of the enzyme in the presence of 0.05 M imidazole (pH 6.5) after dialysis for 48 h at 4 °C followed by incubation for 10 min at 45, 55, 65, and 77 °C. In the absence of glycerol, there was rapid loss of activity. However, in the presence of glycerol, purified enzyme has been kept at -20 °C for more than 4 years without any loss of activity. Imidazole appears to be the most protective buffer examined to date. HEPES, ACES, MES, and Tris-glycine have almost the same relative protective effect as imidazole (optimum pH for stability is pH 6.0-6.5). However, phosphate is considerably less protective and is no longer used as the buffer for the purification of B. subtilis glucose dehydrogenase (23).

The amount of glycerol required to effect protection of the enzyme activity also depends on the buffer; less glycerol was required to effect the same protection when imidazole was used as the buffer (Fig. 1) than when phosphate was used. Relatively large amounts of glycerol (i.e. 20% (w/v) in imidazole buffer) were required to assure protection during enzyme purification; this is consistent with Gekko and Timasheff's proposal (33, 34) that glycerol's protective effect acts via a "general and nonspecific effect".

**Substrate Specificity of Spore Germination and Glucose Dehydrogenase**—In the presence of glucose, B. subtilis spores, which have been heat-shocked and incubated in the presence of potassium, fructose, and asparagine, begin spore germination and enter vegetative growth (10, 11). The rate of entry into the initial stage in the spore germination (phase darkening) can be studied spectrophotometrically (determining the decrease in absorbance at 625 nm of resuspended B. subtilis spores), and it is possible from the determination of the maximum rate of phase darkening as a function of glucose concentration to obtain an apparent $K_m$ of glucose for spore phase darkening (Table II). Similar $K_m$ values can also be obtained for 2-deoxyglucose and glucosamine (35) and can be compared to the in vitro $K_m$ values observed for these same substrates with glucose dehydrogenase at an assay pH of 6.5 (Table II).

A putative role of glucose dehydrogenase in glucose spore germination is made somewhat more tenable because of the observation that the high $K_m$ values for glucose previously reported for this enzyme (23) at the conventional assay pH values (pH 7.8–8.0) are substantially reduced when the pH used for the assay (Table II) is the same as the intracellular pH found in the dormant spores (pH 6.3–6.4 for Bacillus cereus and Bacillus megaterium spores) (36). Fig. 2 shows the effect of pH on the apparent $K_m$ and $V_{max}$ of the B. subtilis

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**TABLE I**

| Purification of B. subtilis glucose dehydrogenase | Total enzyme units | Total protein | Specific activity | Yield |
|-------------------------------------------------|-------------------|--------------|------------------|-------|
| µmol/min at 25 °C                              | µg/mg | %             |                  |       |
| Cell-free extract                              | 4,130 | 14,000        | 0.295            | 100   |
| After DEAE-Sephacel                            | 2,660 | 2,000         | 1.33             | 64    |
| After gel filtration                           | 2,280 | 1,700         | 1.34             | 55    |
| After hydroxyapatite                           | 2,120 | 809           | 2.64             | 51    |
| After second gel filtration                    | 1,850 | 246           | 7.52             | 45    |
| After second hydroxyapatite                    | 1,730 | 18.5          | 93.5             | 42    |
| After hydrophobic                               | 1,130 | 3.8           | 298              | 27    |
| After ω-aminohexyl-Sepharose                    | 802   | 2.2           | 364              | 19    |

* A glucose dehydrogenase enzyme unit assayed at room temperature (23 °C) corresponds to 2.5 units at 37 °C. (Thus, 368 µmol/min/mg of protein at 23 °C is equivalent to 920 µmol/min/mg of protein at 37 °C.)

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5 R. Ramaley, unpublished observations.

**FIG. 1.** Glucose dehydrogenase activity remaining after dialysis against 0.05 mM imidazole buffer (pH 6.5) containing the indicated concentration of glycerol at 4 °C for 48 h (C) followed by incubation for 10 min at 45 °C (Δ), 55 °C (○), 65 °C (×), and 75 °C (●).
**B. subtilis Glucose Dehydrogenase**

**TABLE II**

| Substrate       | $K_m$ (M) | $V_{max}$ (umol/min/mg) | $V_{max}/K_m$ |
|-----------------|-----------|-------------------------|---------------|
| d-Glucose       | 0.19 ± 0.2| 0.13 ± 0.004            | 100           |
| 2-Deoxyglucose  | 0.76 ± 0.08| 0.12 ± 0.003            | 22            |
| D-Glucosamine   | 4.8 ± 1.8 | 0.09 ± 0.01             | 2.6           |

**DISCUSSION**

Glycerol Protection of B. subtilis Glucose Dehydrogenase—
The use of glycerol to protect B. subtilis glucose dehydrogenase has both practical and interpretive values. It has been possible to use the glycerol protection of the enzyme to purify the glucose dehydrogenase of B. subtilis and B. megaterium to homogeneity. Use of glycerol protection has also clarified some molecular weight discrepancies reported for B. subtilis glucose dehydrogenase. For example, Hachisuka and Tochikubo (38) reported in heated spores of B. subtilis an inactive glucose dehydrogenase ($M_r = 40,000$) that could be activated by incubation with EDTA and dipicolinic acid, etc to $M_r = 100,000$. More recently Yokota et al. (19) reported in a Bacillus isolate BG 1722 an inactive form of glucose dehydrogenase (with a $M_r = 45,000$) which was activated by NAD- and NADP-type compounds producing $M_r = 80,000$. However, gel filtration of the glucose dehydrogenase of the BG 1722 isolate of Yokota et al. (19) (in the presence of 20% (w/v) glycerol in 0.05 imidazole (pH 6.5)) gave only a single molecular specific species of approximately 125,000 (similar to the results observed for the B. subtilis enzyme; Fig. S-11). This and NAD reactivation of B. subtilis glucose dehydrogenase (19, 23) observations are consistent with a hypothesis that, in the absence of glycerol, the B. subtilis glucose dehydrogenase readily dissociates into subunits and that NAD can reverse this dissociation. Incubation of the B. subtilis enzyme at pH 8.5–9.0 at 37 °C in the absence of NAD or glycerol, results in loss of enzymatic activity with an increasingly longer lag time required for NAD reactivation (suggesting that a conformational change may be required prior to reassociation to the active tetramer). The dissociation to subunits at high pH has also been observed with the glucose dehydrogenase from B. megaterium (30), and the dissociation of B. subtilis or B. megaterium glucose dehydrogenase in the absence of NAD or glycerol followed by reassociation under conditions of enzymatic assay (which contains NAD) may explain the additional enzyme forms of glucose dehydrogenase observed following gel filtration (19) or polyacrylamide gel electrophoresis (29, 30) in the absence of glycerol.

Glycerol protection by favoring subunit association via hydrophobic bond stabilization is not unique to sporulation or to Bacillus enzymes but is likely a general phenomenon (33, 34). For example, mammalian glucose-6-phosphate dehydrogenase is stabilized by the presence of NADP (39), but it also can be purified in the absence of NADP, provided glycerol is included in all purification buffers (40, 41). We have purified rat liver glucose-6-phosphate dehydrogenase for comparative purposes and found that although the rat liver enzyme is quite labile in the absence of glycerol, the B. subtilis glucose dehydrogenase is several orders of magnitude more labile which explains why the B. subtilis glucose dehydrogenase was so difficult to purify unless glycerol was present during purification. Hydrophobic stabilization of Bacillus glucose dehydrogenase had been previously postulated by Bach (42) and Sadoff (43) based on their observation that high ionic strength (which also promotes hydrophobic interaction) stabilized the B. cereus glucose dehydrogenase (44), and high ionic strength was also observed to be an important component in the NAD reactivation of the B. subtilis glucose dehydrogenase reported by Fujita et al. (23).

Purification and Properties of the B. subtilis Glucose Dehydrogenase—There was no evidence obtained during purification of the glucose dehydrogenase that there was more than one molecular species of the enzyme prepared from either the mature spores or sporulating cells of B. subtilis (Figs. S-1-S-6) or from B. megaterium spore. Antibody prepared with the

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*R. Ramaley and J. Vary, unpublished observations.*

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*R. Ramaley and K. Barker, unpublished observations.*
B. subtilis glucose dehydrogenase formed a single precipitin line with extracts prepared from mature spore or sporulating cells in double diffusion (micro Ouchterlony) plates (48), and the enzyme in the precipitin line was enzymatically active (provided 20% (w/v) glycerol in 0.05 M imidazole (pH 6.5) was present in the agarose to stabilize the enzyme and unprecipitated enzyme was removed by incubating the Ouchterlony plates in glycerol/imidazole buffer at 4°C overnight prior to incubation with the tetrazolium reaction mixture). There is also no evidence of any precursor or another immunologically reactive form of the spore enzyme synthesized during vegetative growth, and then modified into the spore enzyme as has been proposed for the B. cereus aldolase (46). Extracts of stage 0 mutants of B. subtilis (i.e. mutants unable to start sporulation) have no antibody cross-reactive material present, which is consistent with the B. subtilis glucose dehydrogenase being a sporulation-specific protein.

The purified B. subtilis glucose dehydrogenase has four subunits of M, = 31,500 (Fig. S-9) and appears to have a molecular weight just slightly larger than the M. megaterium glucose dehydrogenase (29). The enzymes purified from the two Bacillus species seem to be quite similar in general physical and catalytic properties, and further studies of the M. megaterium glucose dehydrogenase role in spore germination should be quite informative. B. megaterium spores, unlike B. subtilis spores, will start to germinate (phase darken) upon incubation with glucose without the requirement of an acceptor or another metabolite (47) although the action of M. megaterium glucose dehydrogenase may not be the trigger event (48-50) which results in irreversible cortex hydrolysis (51).

Role of Glucose Dehydrogenase in Spore Germination—Although there is similarity between the carbohydrate specificity for spore germination and the carbohydrate specificity for glucose dehydrogenase (10, 11), the putative participation of glucose dehydrogenase in B. subtilis spore germination remains based on circumstantial evidence, such as that included in the present paper. Definitive proof of involvement will require glucose dehydrogenase mutants which are not available at present. It seems unlikely that metabolism of glucose by glucose dehydrogenase constitutes the initial trigger step in the germination process in B. subtilis, because we have reconfirmed the absolute requirement (10, 11) for the preincubation of heat-shocked B. subtilis spores with potassium, fructose, and asparagine (30 min at 57°C) in order to show the phase darkening of the spores, suggesting that some other binding and metabolic events must be occurring during this preincubation period. In addition, although 2-deoxyglucose is a very efficient substrate for glucose dehydrogenase, it was not quite as effective in producing phase darkening of spores (Table II), suggesting that the initial effect of glucose may require some other metabolic or transport event to facilitate spore phase darkening.

In summary, glycerol protection of B. subtilis glucose dehydrogenase has permitted its purification and its subsequent use in obtaining specific polyclonal antibody prepared in rabbits (52) and monoclonal antibody prepared by the mouse hybridoma technique. 6 The rabbit antibody has recently been used to isolate the gene that codes for the B. subtilis glucose dehydrogenase (52). The current availability of purified enzyme has also permitted an examination of some of the physical and chemical properties of the enzyme, including the terminal amino acid sequence of the enzyme. 7

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6 L. Headricks, M. Heidrick, and R. Ramaley, unpublished observations.
7 S. Rudikoff, personal communication.

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Supplementary Material

**Glycerol Protection and Purification of Bacillus subtilis Glucose Dehydrogenase**

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**EXPERIMENTAL PROCEDURE**

**bacterial and media.** The Bacillus subtilis strain used for the purification of the glucose dehydrogenase was obtained from Ernst Freese (bacterial and media). It was a prototrophic Narbug-68 strain observed by H. N. Subtilis was identified as a stage 5 sporulation blocked isolated from arsenic-resistant Bacillus strain obtained from Ernst Freese, Institute for Fermentation, Osaka, Japan.

The medium used for the growth of the B. subtilis-61297 was nutrient broth (6.51 g/l) grown at the Institute for Fermentation, Osaka, Japan. The medium was supplemented with 0.01 M ethanolamine and 0.1 M potassium phosphate (pH 6.51). The majority of the glucose dehydrogenase activity was removed from the cells by centrifugation at 2,500 rpm, 30 min at 10°C, the cells were harvested by centrifugation and stored at -20°C until used. Yield of the enzyme was 4 g of cells (wet weight) per liter of medium.

**Fractionation of Cell Free Extract.** 500 grams (wet weight) of frozen cells were added to 2 liters of 0.05 M imidazole, pH 6.1, containing 20 g/l glucose dehydrogenase activity, 0.1 M phenylmethyl sulfonyl fluoride (PMSF) and 1.0 M 2-mercaptoethanol. After the cells had thawed, the mixture was adjusted to pH 6.5-7.0 by the addition of 1.0 M imidazole. The enzyme was added (1.0, 0.4 M/l). The mixture was brought to 38°C and kept at this temperature for 30 min by which time all of the cells had lost their phase dark appearance upon phase contrast microscopical observation.

The mixture was chilled to 4°C and sonicated (heat system modifier) for one minute intervals for a total of 30 min for the time the mixture had lost its phase dark appearance. Then all of the cells had lost their phase dark appearance upon phase contrast microscopical observation.

**Column Chromatography of Bacillus subtilis**

Two liters of DEAE Sephacel was added to the 20 ml of a liter by liter buffer (pH 6.51) containing 20% glycerol, 200 g/l glucose dehydrogenase activity (w/v) and 0.1 M 2-mercaptoethanol. The AMPSI was added (1.0, 0.4 M/l). The mixture was brought to 38°C and kept at this temperature for 30 min by which time all of the cells had lost their phase dark appearance upon phase contrast microscopical observation. The binding of the enzyme to the DEAE-Sepharose was determined considerable viscous material.

The DEAE Sepharose Bound enzyme was added by addition of 500 ml of 0.01 M imidazole buffer containing 20 g/l glucose dehydrogenase activity, 0.1 M 2-mercaptoethanol and 1.0 M potassium phosphate (pH 6.51) containing 200 g/l of DEAE Sepharose equilibrated by the addition of 20 g/l glucose dehydrogenase activity and 1.0 M potassium phosphate (pH 6.51) containing 0.1 M 2-mercaptoethanol. The enzyme was eluted with a sharp two-liter linear gradient of 0.01 to 0.6 M imidazole buffer (pH 6.1) containing 20 g/l glucose dehydrogenase activity (w/v) and 0.1 M 2-mercaptoethanol. The enzyme eluted at a single isoelectric point except for the major band of pigmented proteins (elution profile not shown).

**Hydrophobic Column Chromatography.** The fractions containing the majority of the glucose dehydrogenase activity were combined and applied to a 2.5 x 20 cm column of hydrophobic column chromatography (Figure 5-2). The absorption at 280 nm (0.1) glucose dehydrogenase activity (l), conductivity (l), and potassium phosphate concentration (l) were determined for the indicated fractions.

**Hydrophobic Column Chromatography.** The fractions containing the majority of the glucose dehydrogenase activity were combined and applied to a 2.5 x 20 cm column of hydrophobic column chromatography (Figure 5-3). The absorption at 280 nm (0.1) glucose dehydrogenase activity (l), conductivity (l), and potassium phosphate concentration (l) were determined for the indicated fractions.

**N-linked Glycoprotein Column Chromatography.** The fractions containing the majority of the glucose dehydrogenase activity were eluted with a decreasing linear gradient of 0.0 to 0.5 M ammonium sulfate in the imidazole/glcyerol buffer (Figure 5-5).

**Reduction Hydroxyapatite Column Chromatography.** The fractions containing the majority of the glucose dehydrogenase activity (fraction 38 to 46) were combined and applied to a 1.5 x 20 cm column of hydroxyapatite (LKB). The enzyme was eluted with a sharp two-liter linear gradient of 0.0 to 0.5 M ammonium sulfate in the imidazole/glcyerol buffer (Figure 5-5). From this point, all of the fractions in the major protein present as determined by polyacrylamide gel electrophoresis (PAGE).

**FIGURE**

**Figure 5-1.** Hydroxyapatite (LKB HA) column chromatography of B. subtilis glucose dehydrogenase. The absorbance at 280 nm (w), glucose dehydrogenase activity (l), conductivity (l) and potassium phosphate concentration (l) were determined for the indicated fractions.

**Figure 5-2.** Sephacryl S-300 column chromatography of B. subtilis glucose dehydrogenase. The absorbance at 280 nm (0.1) glucose dehydrogenase activity (l), conductivity (l) and potassium phosphate concentration (l) were determined for the indicated fractions.

**Figure 5-3.** Hydroxyapatite (LKB HA) column chromatography of B. subtilis glucose dehydrogenase. The absorbance at 280 nm (l), glucose dehydrogenase activity (l), conductivity (l) and potassium phosphate concentration (l) were determined for the indicated fractions.
B. subtilis Glucose Dehydrogenase

Figure 6a. Chromatography of B. subtilis glucose dehydrogenase. The protein (0.01 mg), glucose dehydrogenase activity (0.18 mU) and potassium chloride concentration (0.10 M) were determined for the individual fractions.

Chromatography. The peak fractions (fractions 7 to 12) were combined and concentrated to 1.5 ml using a Millipore CN-10C membrane filter, dialyzed against one liter of phosphate/glycine buffer and applied to a 1 x 10 cm column of DEAE Sepharose Fast Flow (Pharmacia) equilibrated with 0.02 M imidazole/glycine buffer. The enzyme was eluted with 200 ml of a linear 0-50 mM potassium chloride gradient in 0.1 M phosphate (pH 7.0) containing 0.1 M glycine.

Figure 6b. Chromatography of B. subtilis glucose dehydrogenase. The protein (0.01 mg), glucose dehydrogenase activity (0.18 mU) and potassium chloride concentration (0.10 M) were determined for the individual fractions.

This pH was identical to the isoelectric point previously obtained by column isoelectric focusing of apo glucose dehydrogenase (55) in pH 4-6 polyacrylamide gels (56). The fractions containing the majority of the glucose dehydrogenase activity (fractions 7 to 12) were combined and concentrated to 1.5 ml. The glucose dehydrogenase activity (0.18 mU) and potassium chloride concentration (0.10 M) were determined for the individual fractions.

Figure 6c. Glucose dehydrogenase elution from DEAE Sepharose Fast Flow. The enzyme was eluted with a linear 0-50 mM potassium chloride gradient in 0.1 M phosphate (pH 7.0) containing 0.1 M glycine.

Figure 6d. Glucose dehydrogenase elution from DEAE Sepharose Fast Flow. The enzyme was eluted with a linear 0-50 mM potassium chloride gradient in 0.1 M phosphate (pH 7.0) containing 0.1 M glycine.

Figure 6e. Glucose dehydrogenase elution from DEAE Sepharose Fast Flow. The enzyme was eluted with a linear 0-50 mM potassium chloride gradient in 0.1 M phosphate (pH 7.0) containing 0.1 M glycine.

Figure 6f. Glucose dehydrogenase elution from DEAE Sepharose Fast Flow. The enzyme was eluted with a linear 0-50 mM potassium chloride gradient in 0.1 M phosphate (pH 7.0) containing 0.1 M glycine.

Figure 6g. Glucose dehydrogenase elution from DEAE Sepharose Fast Flow. The enzyme was eluted with a linear 0-50 mM potassium chloride gradient in 0.1 M phosphate (pH 7.0) containing 0.1 M glycine.

Figure 6h. Glucose dehydrogenase elution from DEAE Sepharose Fast Flow. The enzyme was eluted with a linear 0-50 mM potassium chloride gradient in 0.1 M phosphate (pH 7.0) containing 0.1 M glycine.

Figure 6i. Glucose dehydrogenase elution from DEAE Sepharose Fast Flow. The enzyme was eluted with a linear 0-50 mM potassium chloride gradient in 0.1 M phosphate (pH 7.0) containing 0.1 M glycine.

Figure 6j. Glucose dehydrogenase elution from DEAE Sepharose Fast Flow. The enzyme was eluted with a linear 0-50 mM potassium chloride gradient in 0.1 M phosphate (pH 7.0) containing 0.1 M glycine.

Figure 6k. Glucose dehydrogenase elution from DEAE Sepharose Fast Flow. The enzyme was eluted with a linear 0-50 mM potassium chloride gradient in 0.1 M phosphate (pH 7.0) containing 0.1 M glycine.

Figure 6l. Glucose dehydrogenase elution from DEAE Sepharose Fast Flow. The enzyme was eluted with a linear 0-50 mM potassium chloride gradient in 0.1 M phosphate (pH 7.0) containing 0.1 M glycine.

Figure 6m. Glucose dehydrogenase elution from DEAE Sepharose Fast Flow. The enzyme was eluted with a linear 0-50 mM potassium chloride gradient in 0.1 M phosphate (pH 7.0) containing 0.1 M glycine.

Figure 6n. Glucose dehydrogenase elution from DEAE Sepharose Fast Flow. The enzyme was eluted with a linear 0-50 mM potassium chloride gradient in 0.1 M phosphate (pH 7.0) containing 0.1 M glycine.

Figure 6o. Glucose dehydrogenase elution from DEAE Sepharose Fast Flow. The enzyme was eluted with a linear 0-50 mM potassium chloride gradient in 0.1 M phosphate (pH 7.0) containing 0.1 M glycine.

Figure 6p. Glucose dehydrogenase elution from DEAE Sepharose Fast Flow. The enzyme was eluted with a linear 0-50 mM potassium chloride gradient in 0.1 M phosphate (pH 7.0) containing 0.1 M glycine.

Figure 6q. Glucose dehydrogenase elution from DEAE Sepharose Fast Flow. The enzyme was eluted with a linear 0-50 mM potassium chloride gradient in 0.1 M phosphate (pH 7.0) containing 0.1 M glycine.

Figure 6r. Glucose dehydrogenase elution from DEAE Sepharose Fast Flow. The enzyme was eluted with a linear 0-50 mM potassium chloride gradient in 0.1 M phosphate (pH 7.0) containing 0.1 M glycine.

Figure 6s. Glucose dehydrogenase elution from DEAE Sepharose Fast Flow. The enzyme was eluted with a linear 0-50 mM potassium chloride gradient in 0.1 M phosphate (pH 7.0) containing 0.1 M glycine.

Figure 6t. Glucose dehydrogenase elution from DEAE Sepharose Fast Flow. The enzyme was eluted with a linear 0-50 mM potassium chloride gradient in 0.1 M phosphate (pH 7.0) containing 0.1 M glycine.

Figure 6u. Glucose dehydrogenase elution from DEAE Sepharose Fast Flow. The enzyme was eluted with a linear 0-50 mM potassium chloride gradient in 0.1 M phosphate (pH 7.0) containing 0.1 M glycine.

Figure 6v. Glucose dehydrogenase elution from DEAE Sepharose Fast Flow. The enzyme was eluted with a linear 0-50 mM potassium chloride gradient in 0.1 M phosphate (pH 7.0) containing 0.1 M glycine.
B. subtilis Glucose Dehydrogenase

0.068 mg of purified glucose dehydrogenase was added to 1.0 ml column of Sepharl 3-500 previously equilibrated with 0.05 M phosphate (pH 7.4), 200 mM glucose, 0.1 M NaF, and 0.1 M 2-mercaptoethanol and eluted with the same buffer (Figure 6-12). The other protein standards were applied individually and horse heart cytochrome C was used as an internal standard. 0.020 mg of B. subtilis glucose dehydrogenase, purified from spore extract supernatant (10) by the same procedure, was also applied to the same column and eluted slightly later than did the B. subtilis enzyme (not shown) (116,000 vs 120,000).

Determination of the Stability of glucose dehydrogenase. 1.5 mg of purified enzyme was placed in the activity of the indicated buffers with or without glucose, as indicated. These samples were placed in 9 mm test tubes, incubated and dialyzed for the specified time at 2°C. The samples were removed from the dialysis tubing and the amount of glucose dehydrogenase activity remaining was determined.

Enzyme Assay. The B. subtilis and B. megaterium glucose dehydrogenase activities were measured by the increase in absorbance at 340 nm (glucose reductase) or decrease at 346 nm (glucose oxidase) in the presence of glucose for the determination of enzyme purity. The rat liver glucose-6-phosphate dehydrogenase activity was assayed at 37 °C in a cuvette containing 1% glucose-6-phosphate, 100 mM NaF, and 100 mM Tris-HCl (pH 8.0).

Protein was determined by the Comassie Blue dye binding assay of Bradford (58) in a volume of one milliliter, and the samples were diluted so as to be near the midpoint for the standard protein curve (110 mg of protein). Bovine serum albumin (fraction V) was used as the protein standard.

Determination of A600 Phase Darkening upon Addition of Glucose. Spores of the B. subtilis 60015-1373 grown on nutrient broth-10% agar plates were washed with tap water, and washed seven times with cold distilled water until they were free of visible contaminates. The spores were incubated at 25 °C for 20 minutes and discarded the supernatant until there were no vegetative cells present. The spores were stored at -20 °C until used. A 2% solution of glucose was added to a 0.1 M potassium phosphate buffer (pH 7.2) at an approximate 1:100 spore concentration and stored at 0 °C until used (not more than four hours). The spores were incubated at 65 °C for 30 minutes at pH 7.2 with 100 mM sodium phosphate buffer (pH 7.2). The incubated spores were added to the enzyme solution at the concentration shown and the decrease in absorbance at 600 nm determined. The rate of decrease shown and used for the calculation of Ae is the maximal rate of decrease observed following addition of substrate. The initial rate of decrease because there was a lag phase before darkening begins (10,113). No decrease in absorbance was observed in the absence of added substrate.

Materials

Chemicals and Biochemicals. Glucose and inorganic chemicals were obtained from Fisher Scientific Company. All biochemicals not otherwise specified were obtained from Sigma Chemical Company. NAD and other chemicals were obtained from P.L. Biochemicals. Media components were obtained from Difco Laboratories. Water used to make up solutions was deionized and the water used to prepare saline equilibrated prior to use.
Glycerol protection and purification of Bacillus subtilis glucose dehydrogenase.
R F Ramaley and N Vasantha

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