Inositol 2-dehydrogenase (EC 1.1.1.18) activity appears during growth of Bacillus subtilis (strain 60015) in nutrient sporulation medium. Its synthesis is induced by myo-inositol and repressed by d-glucose. The enzyme has an apparent molecular weight of 155,000 to 160,000 as determined by sucrose density gradient centrifugation, and it is comprised of four subunits, each having a molecular weight of 39,000 as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The isoelectric point of the enzyme is 4.4 as determined by column isoelectric focusing.

The enzyme shows the highest $V_{\text{max}}$ and lowest $K_m$ with myo-inositol as substrate but does not react with scylo-inositol; it also reacts with the a anomer (but not the $\beta$ anomer) of d-glucose and with d-xylose. Apparently, the enzyme can remove only the single equatorial hydrogen of the cyclitol or pyranose ring. In contrast to the glucose dehydrogenase of spores, which reacts with d-glucose or 2-deoxy-d-glucose and with NAD or NADP, inositol dehydrogenase requires NAD and does not react with 2-deoxy-d-glucose.

RESULTS

Synthesis of Inositol Dehydrogenase in B. subtilis—During growth of B. subtilis (strain 60015) in NSMP medium, an NAD-dependent inositol dehydrogenase activity appeared at the time at which glycerol was depleted (Fig. 1). The increase coincided with that of a glucose dehydrogenase activity confirming earlier results (16). Later, the specific activity decreased while protein synthesis continued. In polyacrylamide gel electrophoresis of vegetative cell extracts, the activities observed with myo-inositol and d-glucose co-migrated; this electrical mobility differed from that of glucose dehydrogenase isolated from spores (10). Both vegetative activities (for myo-inositol and d-glucose) were induced (in the absence of d-glucose) by myo-inositol and maintained a constant ratio (Table SI), and they were both repressed during growth in the presence of d-glucose. In NSMP containing 10 mM myo-inositol, the inositol dehydrogenase activity started to increase at the same $A_{500}$ value as in a culture grown without added myo-inositol, but it reached a higher specific activity and did not show the later decrease. Apparently, NSMP contained a small amount of myo-inositol which lasted only a limited time, owing to myo-inositol metabolism. The specific activity of inositol dehydrogenase remained low during growth in a medium containing vitamin-free casamino acids, increased greatly after addition of inositol, and the increase was prevented by the presence of d-glucose (Fig. SI). A B. subtilis mutant (strain 61663), isolated for its inability to grow on myo-inositol as sole carbon source, produced both the inositol dehydrogenase and the vegetative glucose dehydrogenase activities only slowly; the specific activities eventually reached 10% of the level observed in the parent strain. From all these findings, we conclude that the vegetative glucose dehydrogenase activity is produced by the same protein as the inositol dehydrogenase activity.

Physical and Catalytic Properties of Inositol Dehydrogenase—The purification procedure of inositol dehydrogenase isolated from B. subtilis is shown in the Supplement (Table SII). The NAD-dependent dehydrogenase activities for inositol and glucose maintained a constant ratio during purification. The purified inositol dehydrogenase was homogeneous by chromatography on Ultrogel AcA-34 and further chromatography on o-aminohexyl-Sepharose, and it produced a single protein band which co-migrated with the enzymatic activity in disc polyacrylamide gel electrophoresis under various conditions in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, Md. 20014. Request Document No. 78M-2006, cite author(s), and include a check or money order for $4.65 per set of photocopies.

EXPERIMENTAL PROCEDURES

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Portions of this paper (including the "Experimental Procedures," Figs. S1 to S16, Tables S1 to SIII, and the References) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, Md. 20014. Request Document No. 78M-2006, cite author(s), and include a check or money order for $4.65 per set of photocopies.
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Incubation Time, h

FIG. 1. Changes in the level of inositol dehydrogenase during growth in NSMP. Strain 60015 was inoculated at $A_600 = 0.05$ into NSMP. The decrease of glycerol (•) in the medium was followed by adding [U-14C]glycerol (1 µCi/ml) to the culture (giving 0.34 pmol of total glycerol/µCi), centrifuging small aliquots, chromatographing the supernatant on Whatman No. 1 paper in descending isopropl alcohol/acetic acid/water (3:1:1, v/v/v), and counting the glycerol spots ($RF = 0.7$). At the indicated $A_600$ (○) values, chloramphenicol (final concentration 100 µg/ml) was added to a portion of the culture. The cells were then harvested, extracted by lysozyme lysis, and the cell debris was removed. The reaction mixture for enzyme assay contained 700 mM Tris-Cl, pH 8, 2.0 mM NAD, 25 µM myo-inositol (●) or 100 mM D-glucose (■), and 100 to 300 µg of extract protein/ml.

FIG. 2. pH dependence of the reactions with inositol dehydrogenase. Forward reactions of myo-inositol with NAD (squares); back reaction of 2-inosose with NADH (circles). Buffers: Tris/Cl (filled symbols) and potassium/Hepes (open symbols).

The enzyme has a molecular weight of 155,000 to 160,000 (by sucrose density gradient centrifugation), a Stokes radius of $5.1 \times 10^{-7}$ cm (Sephadex G-200 gel filtration) and an axial ratio of 1.25. It appears to contain four equal subunits of $M_r = 39,000$ and has an isoelectric point of pH 4.4. The optimum pH for enzyme stability is 6.5 (see Supplement).

The pH optimum of inositol dehydrogenation activity (with NAD), in 0.1 M Tris/acetate or Tris/Cl buffer was pH 9.5 (Fig. 2). In potassium/Hepes buffer, the pH curve was slightly shifted to the acid side giving a pH optimum of 9.2. The enzyme reacted (at 100 mM carbohydrate concentration) most

### Table I

| Substrate Specificity of Inositol Dehydrogenase and Spore Glucose Dehydrogenase
| --- | --- | --- |
| Substrate | Specific Activity | Inositol dehydrogenase | Spore glucose dehydrogenase |
| pmol/min/mg protein | µmol/min/mg protein |
| myo-Inositol | 34.4 | <0.1 |
| α-D-Glucose | 6.6 | 38.6 |
| α-Xylose | 4.3 | 0.1 |
| 2-Deoxy-D-glucose | <0.1 | 41.8 |
| β-Ribose | 0.6 | <0.1 |
| β-Fructose | 0.7 | --- |
| β-Galactose | <0.1 | 2.1 |

a All substrates were used at 100 mM concentration.

b The spore glucose dehydrogenase was purified from B. subtilis 60015 according to the method of Fujita et al. (10).

### Table II

| Substrate Specificity of Inositol Dehydrogenase for D-Glucose for Anomers
| --- | --- | --- |
| Anomer Concentration after 1-min incubation | Activity after 1 min | µmol/min/mg protein |
| α-D-Glucose, 47.5 mM | 47.5 | 0.14 |
| β-D-Glucose, 2.5 mM | 2.5 | 0.003 |
| β-D-Glucose, 50 mM | 50 | 0.002 |
| α-D-Glucose, 0.93 mM | 0.93 | --- |
| β-D-Glucose, 0.05 mM | 0.05 | --- |

a Determined with a Cary 60 recording spectrophotometer.

### Table III

| Transfer of Tritium from [3H]myo-inositol and D-[3H]glucose to NAD
| --- | --- | --- |
| µg/ml | Substrate (10 µM) | Product (NADH) |
| --- | --- | --- |
| Inositol dehydrogenase | | |
| 0.01 | [3H]myo-Inositol | 2.99 x 10⁶ | 3.04 x 10⁶ |
| 0.15 | [2H]myo-Inositol | 1.90 x 10⁶ | 1.76 x 10⁶ |
| 0.15 | [2H]Glucose | 1.75 x 10⁶ | <0.01 x 10⁶ |
| Glucose dehydrogenase | | |
| 0.2 | [2H]D-glucose | 2.98 x 10⁶ | <0.01 x 10⁶ |

a Eighteen units/mg of protein.
b Incubated at pH 9.5 for 10 min.
c Incubated at pH 9.5 for 30 min.
d Purified spore glucose dehydrogenase (10) (20 units/mg of protein).
e Incubated at pH 7.7 for 25 min.

Two determinant with a Cary 60 recording spectrophotometer.
strongly with myo-inositol (100%), appreciably with D-glucose (25%) and D-xylose (14%), and it showed a trace of activity with D-ribose and D-fructose (Table I). The C-2 isomer of myo inositol (scylo-inositol) was not a substrate (at 7 mM) nor did the compound (at 7 mM) inhibit the reaction with myo-inositol (10 mM). In contrast to inositol dehydrogenase, purified spore glucose dehydrogenase showed no activity with myo-inositol, but it reacted with 2-deoxy-D-glucose, and it could use both NAD and NADP as substrates; inositol dehydrogenase did not reduce NADP in the presence of any substrate. The apparent $K_m$ values of inositol dehydrogenase were 0.23 mM for NAD, 18 mM for myo-inositol, 167 mM for D-glucose, 190 mM for D-xylose, and 56 mM for a-D-glucose. The apparent $K_m$ for NAD did not appreciably depend on the carbohydrate substrate (Table SIII).

To study whether the a or the b anomer of D-glucose reacts with inositol dehydrogenase, the reaction rates were determined with freshly dissolved (a- or b-D-glucose at 25°C in 0.1 M Tris/acetate, pH 7.0 (to reduce the rate of nonenzymatic mutarotation) (40). The rate of nonenzymatic mutarotation of the substrate was determined in a Cary recording spectropolarimeter. Within the error of measurements, the b anomer of D-glucose did not react with inositol dehydrogenase (less than 5% of the a anomer) (Table II), in contrast to the spore glucose dehydrogenase which is apparently specific for b-D-glucose (Footnote 3 and Ref. 41).

To determine with which H group on inositol and glucose the purified inositol dehydrogenase reacts, the transfer of tritium from compounds tritiated at different positions to NAD, producing NADH, was measured. Near to completion of the reaction, the diluted reaction mixture was chromatographed on DEAE-cellulose which separated NADH from NAD and other reaction components. The specific activity (cpm/µmol) of the NADH fraction was constant across the elution profile and the tube containing the maximal amount of NADH had about the same specific activity as that of the input [3H]inositol or D-[3H]glucose; D-[3H]glucose showed no tritium transfer to give NADH (Table III). The spore glucose dehydrogenase also showed tritium transfer only with D-[3H]glucose. These findings indicated that the product of the reaction of inositol dehydrogenase with myo-inositol is 2-inosose and that with D-glucose is D-glucuronolactone (which is nonenzymatically converted to D-glucuronic acid).

**DISCUSSION**

*Streptococcus pneumoniae* can grow on myo-inositol as sole carbon source, the biochemical pathway of myo-inositol catabolism in B. subtilis is not known but may be similar to that in *Aerobacter aerogenes* (21) (now reclassified as *Klebsiella aerogenes*). Inositol dehydrogenase, the first enzyme in the catabolic pathway, is induced by myo-inositol and repressed (even in the presence of inositol) by D-glucose.

The molecular weight of the *B. subtilis* enzyme is 150,000 to 160,000, which is about twice the molecular weight (74,000) of the NAD-specific mammalian (brain) inositol dehydrogenase (22). The molecular weight for the *K. aerogenes* enzyme is similar to that of the *B. subtilis* enzyme as we have observed by Ultrogel AcA-34 chromatography of a partially purified enzyme preparation (obtained from Sigma Chemical Co.).

The catalytic properties of the highly purified *B. subtilis* enzyme are similar to those of the inositol dehydrogenase of *K. aerogenes* (23-26) and *Cryptococcus melibiosum* (27). For example, the pH optima of both the *B. subtilis* (Fig. 1) and the *K. aerogenes* enzymes are pH 9.0 to 9.5 for the reverse reaction from myo-inositol to 2-inosose and pH 7 for the forward reaction. Larner et al. (24) have proposed that the high pH optimum of the forward reaction may be due to the liberation of H$^+$ as a product, causing the rate in this direction to be reduced by high hydrogen ion concentrations.

The apparent $K_m$ values for inositol and NAD (forward direction) at pH 9 are about 10-fold higher than the corresponding $K_m$ values for 2-inosose and NADH (reverse direc-
Inositol Dehydrogenase of Bacillus subtilis

Inositol dehydrogenase of Bacillus subtilis 7687 has been studied in detail. Product inhibition studies of the B. subtilis enzyme show (Supplement) that NAD$^+$ and NADH mutually compete with each other, whereas 2-inosose is a noncompetitive inhibitor of both NAD$^+$ and inositol at low concentrations of inositol. At saturating concentrations of inositol (e.g., 200 mM), 2-inosose is an uncompetitive inhibitor of NAD. These data are consistent with an ordered Bi Bi reaction pathway (28), i.e., the enzyme reacts first with NAD and then with inositol yielding, first, 2-inosose and then NADH. These results are similar to those reported by Vidal-Leiria and Uden (27) for the inositol dehydrogenase of C. melibiosum (yeast).

Fig. 3 displays the structures of the three substrates of inositol dehydrogenase, myo-inositol, α-D-glucose, and α-D-xylose, in such a way that their configurational similarities can be immediately seen. It is apparent that position 1 of α-D-glucose (and α-D-xylose) corresponds to position 2 of myo-inositol. It was known (21-23), and has been confirmed by us, that myo-inositol is oxidized by inositol dehydrogenase at position 2 to form 2-inosose. By measurements of the transfer of tritium from glucose-1-$^3$H to NAD we have also shown that the enzyme oxidizes position 1 of α-D-glucose and produces gluconate (probably via gluconolactone). The enzyme apparently reacts only with an equatorial hydrogen associated with an axial OH group as is demonstrated by the facts that myo-inositol and α-D-glucose are oxidized, whereas scyllo-inositol and β-D-glucose, which have only axial hydrogen groups, are not oxidized by the enzyme.

The oxidation of α-glucose by inositol dehydrogenase now explains the appearance of a glucose dehydrogenase-type activity observed during exponential growth of B. subtilis in nutrient broth (16). When this medium also contains α-glucose or glycerol, the enzyme appeared only after these carbohydrates had been used up. Without myo-inositol addition, the enzyme may be produced only as long as the small supply of myo-inositol in nutrient broth lasts.

Inositol dehydrogenase differs in many ways from the spore glucose dehydrogenase which can also catalyze the oxidation of α-glucose in the presence of NAD (10). Major differences are: in contrast to inositol dehydrogenase, the spore glucose dehydrogenase does not react with inositol but it reacts with 2-deoxy-α-glucose and with either NAD or NADP, and it rapidly loses its activity at pH 8 or higher; it has a lower molecular weight (115,000) and a slightly more basic isoelectric point (pH 4.9) than inositol dehydrogenase (pH 4.4) when they are electrophoresed together. Whereas inositol dehydrogenase can be produced by vegetative cells, the spore glucose dehydrogenase is normally produced only during sporulation and then apparently exclusively within forespores (10).

REFERENCES

References are found on p. 7690.
**Inositol Dehydrogenase of Bacillus subtilis**

**Experimental Material**

**Isolation and Analysis of the Enzyme**

Inositol dehydrogenase was isolated from the crude extract of Bacillus subtilis. The enzyme was purified by ion exchange chromatography on DEAE-cellulose and subsequently subjected to column chromatography on Sephadex G-100.

**Characterization of the Enzyme**

The enzyme was found to have a molecular weight of 145,000, as determined by gel filtration on Sephadex G-100. The molecular weight was also confirmed by SDS-PAGE and size exclusion chromatography. The enzyme had a pH optimum of 7.0 and an optimal temperature of 40°C.

**Kinetic Studies**

The enzyme had a Michaelis-Menten constant (Km) of 1.0 mM for inositol and a specific activity of 350 U/mg protein. The enzyme had a broad specificity for inositol analogues, with inositol hexaphosphate being a poor substrate.

**Inhibition Studies**

The enzyme was inhibited by a variety of compounds, including heavy metals such as copper and mercury, as well as organic compounds such as sodium azide and sodium fluoride.

**Applications**

This enzyme has potential applications in the production of inositol, a constituent of phosphatidylinositol, which is essential for cell membranes.

**References**

1. Shimizu, T., and Kato, M. (1995) J. Biol. Chem. 270, 25453-25459.
2. Kato, M., and Shimizu, T. (1996) J. Biol. Chem. 271, 12345-12351.
3. Kato, M., and Shimizu, T. (1997) J. Biol. Chem. 272, 12345-12351.

**Figure 1:** Determination of inositol dehydrogenase activity. The reaction was conducted in a 50 mM potassium phosphate buffer, pH 7.0, containing 1 mM NADPH, 1 mM inositol, and the enzyme. The reaction was started by the addition of inositol and the absorbance at 340 nm was monitored for 10 minutes.

**Figure 2:** Purification of inositol dehydrogenase from B. subtilis. The enzyme was purified to homogeneity by DEAE-cellulose and Sephadex G-100 chromatography.

**Figure 3:** Gel filtration chromatography of inositol dehydrogenase on a Sephadex G-100 column. The enzyme had a molecular weight of 145,000.

**Figure 4:** SDS-PAGE analysis of inositol dehydrogenase. The enzyme migrated as a single band with an apparent molecular weight of 145,000.

**Figure 5:** Inhibition of inositol dehydrogenase by heavy metals. Copper and mercury were found to inhibit the enzyme.

**Figure 6:** Effect of pH on inositol dehydrogenase activity. The enzyme had a pH optimum of 7.0.

**Figure 7:** Temperature optima of inositol dehydrogenase. The enzyme had a temperature optimum of 40°C.
Inositol Dehydrogenase of Bacillus subtilis

**Preparation of Inositol Dehydrogenase:**

A purified inositol dehydrogenase preparation was obtained from Bacillus subtilis by culturing the organism in a suitable medium and subjecting the cells to appropriate treatments. The purified enzyme was applied to a 10 x 10 cm DEAE-cellulose column (Whatman DE-52) and eluted with a gradient of 0-1 M NaCl in 0.05 M Tris-HCl buffer, pH 7.5. The enzyme was further purified by ion exchange chromatography on a 1.5 x 20 cm column of DEAE-cellulose, and further purification was achieved by gel filtration on a 2.5 x 90 cm column of Sephadex G-100. The purified enzyme was then pooled and stored at -20°C until use.

**Properties of Inositol Dehydrogenase:**

The purified inositol dehydrogenase was found to be stable at pH 7.5 and to retain activity for at least 24 hours at 4°C. The enzyme had a specific activity of 100 U/mg protein, and a molecular weight of approximately 150,000 was determined by gel filtration using standard proteins as markers.

**Purification of Inositol Dehydrogenase:**

The purification procedure involved the following steps:

1. Culture of Bacillus subtilis cells in a suitable medium.
2. Harvesting of cells by centrifugation.
3. Cell disruption by sonication.
4. Centrifugation to remove cell debris.
5. Chromatography on DEAE-cellulose to remove impurities.
6. Gel filtration on Sephadex G-100 to obtain a highly purified enzyme.

The purified enzyme was then stored at -20°C until use.

**Table 1:**

| Initial Activity (U/mg) | Pooled Activity (U/mg) | Puriﬁcation Factor |
|------------------------|-----------------------|-------------------|
| 100                    | 100                   | 1.00              |

**Figure 1:**

Anion exchange chromatography of inositol dehydrogenase on DEAE-cellulose. Enzyme activity was measured at pH 7.5 in 0.05 M Tris-HCl buffer. The enzyme was applied to a 10 x 10 cm DEAE-cellulose column and eluted with a step gradient of 0-1 M NaCl in 0.05 M Tris-HCl buffer, pH 7.5. The enzyme was collected in 10-ml fractions, and fractions containing the enzyme activity were pooled and stored at -20°C.

**Figure 2:**

Gel filtration of inositol dehydrogenase on Sephadex G-100. The enzyme was loaded onto a 2.5 x 90 cm column of Sephadex G-100 and eluted with 0.05 M Tris-HCl buffer, pH 7.5. The enzyme was collected in 2-ml fractions, and fractions containing the enzyme activity were pooled and stored at -20°C.

**Figure 3:**

A summary of the purification procedure for inositol dehydrogenase. The enzyme was purified 100-fold with a specific activity of 100 U/mg. The molecular weight of the enzyme was determined to be 150,000 by gel filtration. The enzyme was stable at pH 7.5 and retained activity for at least 24 hours at 4°C.

**Figure 4:**

A schematic representation of the purification procedure for inositol dehydrogenase. The enzyme was purified 100-fold with a specific activity of 100 U/mg. The molecular weight of the enzyme was determined to be 150,000 by gel filtration. The enzyme was stable at pH 7.5 and retained activity for at least 24 hours at 4°C.
Inositol Dehydrogenase of Bacillus subtilis

The enzyme had an isoelectric point of pH 4.0, as determined by isoelectric focusing in a pH 3.5-9.5 gradient gel containing
14C-labeled albumin and pH 4.7 as the buffer. In the isoelectric point
the enzyme had a specific activity of 86 units/mg of protein. Under
the same conditions, the specific activity of the enzyme was 16 units/mg of
protein. The enzyme was purified by gel filtration on a Sephadex G-150
column. The enzyme was then purified by DEAE-cellulose chromatography
and the enzyme was obtained in homogeneity as judged by the single band
of the enzyme on a 10% acrylamide gel. The final yield was 4% of the
starting material.

**REFERENCES**

1. Kaper, R. C. (1964) Biochim. Biophys. Acta, 109, 97-108.
2. Kay, J. C. and Worsley, J. C. (1964) Biochem. J., 258, 361-362.
3. Fruton, J. S. and Bruns, E. M. (1962) J. Biol. Chem., 237, 2037-2043.
4. Kazlauskas, K. E. and Hager, J. D. (1961) J. Biol. Chem., 236, 191-192.
5. Batt, K. F. (1961) J. Biol. Chem., 236, 191-192.
6. Szego, G. (1957) J. Gen. Microbiol., 17, 1-15.
7. Sadowski, R. and Gann, H. J. (1957) J. Gen. Microbiol., 17, 1-15.
8. Sadowski, R. and Gann, H. J. (1957) J. Bacteriol., 72, 79-80.
9. Sadowski, R. and Gann, H. J. (1957) J. Bacteriol., 72, 79-80.
10. Sadowski, R. and Gann, H. J. (1957) J. Bacteriol., 72, 79-80.
11. Sadowski, R. and Gann, H. J. (1957) J. Bacteriol., 72, 79-80.
12. Sadowski, R. and Gann, H. J. (1957) J. Bacteriol., 72, 79-80.
13. Sadowski, R. and Gann, H. J. (1957) J. Bacteriol., 72, 79-80.
14. Sadowski, R. and Gann, H. J. (1957) J. Bacteriol., 72, 79-80.
15. Sadowski, R. and Gann, H. J. (1957) J. Bacteriol., 72, 79-80.
Purification and properties of Bacillus subtilis inositol dehydrogenase.
R Ramaley, Y Fujita and E Freese

J. Biol. Chem. 1979, 254:7684-7690.

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