Purification and Characterization of Glucose Dehydrogenase from a Heterotrophically Grown Blue-Green Alga

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ABSTRACT

NADP- and NAD-dependent glucose dehydrogenase was partially purified from a dark-grown blue-green alga (endophytic Nostoc strain MAC). Polyacrylamide gel electrophoresis established that a single protein possessed dual activity for either NADP or NAD. No other electron acceptor substituted for pyridine nucleotides and no evidence for a flavin prosthetic group was found. Although the Km for NADP was 8.8 μM and for NAD 328 μM, the enzyme was equally active with NAD or NADP at saturating levels of substrates. The enzyme was similar to previously described glucose dehydrogenase in that it had a high Km for glucose (18-20 mM at 35 °C) and an alkaline pH optimum of 7.6 to 9.4.

Pyridine nucleotide-dependent glucose dehydrogenase activity was reported for several blue-green algae capable of heterotrophic growth in the dark (8), being most abundant in an endophytic Nostoc sp. isolated from the roots of the cayce Macrozamia lucida (4). The restricted distribution of this enzyme raised the possibility that it played a key role in alleviating a metabolic block to heterotrophic growth in blue-green algae. In order to investigate the function of glucose dehydrogenase in blue-green algal metabolism, it appeared desirable to study the purified enzyme from a species cultured under dark, heterotrophic growth conditions. This paper describes the purification and some properties of glucose dehydrogenase from the Nostoc endophyte strain MAC, grown in the dark on glucose.

MATERIALS AND METHODS

Growth Conditions and Preparation of Enzyme Extracts. Strain MAC was cultured under darkness on medium Cg10 + 0.5% glucose + 0.1% Difco casamino acids, as previously described (8); such dark-grown cells contain about 3 times more glucose dehydrogenase activity than photoautotrophically cultured cells. Cells were harvested and dried over P₂O₅. Cell-free extracts were prepared by making an acetone powder according to the method of Mitsui and Arnon (6). The dried acetone powder was then extracted by sonication in 0.05 M Na, K phosphate, pH 7.6, and soluble and particulate fractions of the extract obtained by centrifugation at 20,000g, as previously reported (8).

Glucose Dehydrogenase Assay and Protein Assay. The stand-
placing the gels in small tubes protected from light (11). The gels were placed in a reaction mixture containing 5 mM glucose, 10 mM MgCl₂·6H₂O, 0.26 mM NADP or 1.25 mM NAD, 0.24 mg/ml phenazine methosulfate, and 0.4 mg/ml nitro blue tetrazolium, all in 50 mM tris-Cl buffer, pH 8.3. A dark purple band appeared after 15 min incubation at ambient temperature which corresponded to purified enzyme stained for protein. Gels placed in tubes lacking substrate did not stain. Gels stained for enzymic activity could be stored indefinitely in 10% trichloroacetic acid.

Characterization of Enzymic Reaction. Km values were calculated from Lineweaver-Burk plots. Reaction rates were taken from the initial, linear 2 to 5 min of the reaction. Except for one experiment where the Km for sugars was determined at 22°C, all kinetic studies and tests of enzyme activity were done at 35°C.

Table 1. Purification Procedure for NADP-linked Glucose Dehydrogenase from Dark-grown Nostoc Strain MAC

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total Activity</th>
<th>Total Protein</th>
<th>Specific Activity</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>20,000g supernatant</td>
<td>128</td>
<td>855</td>
<td>0.15</td>
<td>100</td>
</tr>
<tr>
<td>Ammonium sulfate, 0.3M</td>
<td>111</td>
<td>343</td>
<td>0.33</td>
<td>88</td>
</tr>
<tr>
<td>Saturation</td>
<td>64</td>
<td>27.0</td>
<td>2.32</td>
<td>50</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>60</td>
<td>3.0</td>
<td>21.6</td>
<td>67</td>
</tr>
<tr>
<td>Sephadex G-200</td>
<td>120</td>
<td>855</td>
<td>0.33</td>
<td>88</td>
</tr>
</tbody>
</table>

1 umol NADP reduced/min at 35°C.  
2 Total unit and total protein for entire peak of GPND off Sephadex G-200 column in Fig. I.  
3 Sp. Act. of 35-45 ml fraction from G-200 column in Fig. I.

Alternate electron acceptors were tested in the standard assay system, which contained all components except pyridine nucleotide. Change in absorbance of the following compounds was monitored: dichlorophenol indophenol, 600 nm; nitro blue tetrazolium, 560 nm; ferricyanide, 400 nm; Cyt c, 550 nm; Cyt Csa, 548 nm; FMN and FAD, by scanning absorption spectrum from 350 to 500 nm.

Since pH was found to be a critical factor in testing for modulators of the GPND reaction, all test compounds were adjusted to pH 7.6 prior to addition to the reaction cuvette.

RESULTS

Purification and Properties of the Enzyme. Purification of GPND resulted in an increase of specific activity as shown in Table I. In addition, the fact that the procedure could be performed at room temperature served to remove substantial amounts of protein. Over-all, a 347-fold purification over the 20,000g supernatant was achieved as represented by the enzyme in the peak tubes from Sephadex G-200 chromatography (see below). (NH₄)₂SO₄ was found to be inhibitory to the enzyme after the DEAE-cellulose step, and thus lyophilization or possibly membrane filtration are necessary to concentrate the partially purified enzyme. The rather broad concentration range over which the enzyme eluted from DEAE-cellulose (0.25-1 M phosphate buffer) suggests that the enzyme is quite acidic and adheres strongly to the column.

The elution profile from Sephadex G-200 revealed several points (Fig. 1). First, NADP- and NAD-linked GPND activities appeared in a single symmetrical peak, the ratio of NADP to

Fig. 1. Elution profile for strain MAC glucose dehydrogenase from Sephadex G-200 chromatography. The entire peak of NADP-linked enzyme activity (●—●) was determined, while only selected fractions were analyzed for NAD-linked activity (▲). Change in absorbance at 340 nm was measured in the standard assay.
NAD activity being constant (1:1) across the peak. Second, the major protein eluted from the column before and after the GPND peak. Third, the elution volume for GPND indicated a protein of moderate (medium) mol wt.

The most active enzyme fraction from the Sephadex column (40- to 64-ml fraction) was estimated to be about 50% pure as judged by polyacrylamide gel electrophoresis and activity staining. Samples (50 μg protein) of this purity were loaded onto 6 gels and electrophoresed. One gel was stained for enzyme activity to localize the enzyme. Slices corresponding to the relative mobility of the active enzyme were then made on the other five gels. These small slices were then macerated in a small amount of 50 mM tris-Cl, pH 8.3, and the acrylamide removed by centrifugation. This preparation of enzyme was then electrophoresed and stained for protein and for NADP- and NAD-linked GPND activities. The NADP- and NAD-GPND activities migrated to the same position in the gel, and a single protein band occurred at this position (Fig. 2). Thus, polyacrylamide gel electrophoresis established that the NADP- and NAD-linked GPND activities were due to a single protein with dual activity.

By comparison to the electrophoretic mobility of known protein standards, the mol wt of GPND was estimated to be around 80,000 to 100,000. This mol wt is corroborated by an additional observation: GPND passed readily through a Pellicon membrane (Millipore Co.), type PSJM, of approximately 100,000 mol wt pore size.

The enzyme appeared to lack a flavin moiety. At a concentration of 0.5 mg protein/ml, the purified enzyme from the G-200 40- to 64-ml fraction showed no absorption peaks in the visible spectrum characteristic of flavins, and no fluorescence when treated with acid (pH 2). There was a strong absorption peak at 277 nm, however.

The glucose-dehydrogenase-catalyzed reaction is apparently irreversible at pH 8.5. The purified enzyme showed no ability to convert gluconic acid to glucose with NADPH (0.27 μM) as electron donor at this pH.

The pH optimum for the dehydrogenation ranged from about pH 7.4 to 9.4. Below pH 7, the rate of dehydrogenation dropped off rapidly, such that at pH 6.5, the enzymic rate was only 25% of that at pH 7.6.

The product of the dehydrogenation has been verified as gluconic acid by paper electrophoresis at pH 3.7 in 0.05 M acetate buffer (Fig. 3).

**Reaction Kinetics.** Michaelis constants were calculated for the pyridine nucleotides at 35°C: NADP, 8.8 μM, and NAD, 0.33 mM. Although the Km for both pyridine nucleotides was considerably different, the maximum velocity (Vmax) of the reaction obtained with NAD was the same as that with NADP.

![Fig. 2. Polyacrylamide gels stained for total protein (a) and glucose dehydrogenase activity (NADP-linked [b] and NAD-linked [c]). Gels were electrophoresed and then stained as described in "Materials and Methods." Origin is at top of gels. About 20 μg protein were electrophoresed/gel.](image)

**Fig. 3.** Paper electrophoretogram of components of NADP-dependent glucose dehydrogenase reaction mixture. Purified glucose dehydrogenase from Sephadex G-200 chromatography (20 μg) was allowed to react with 2 mg glucose and 4 mg NADP in 0.02 M tris buffer, pH 7.8, for 8 hr at room temperature, and the reaction terminated by boiling. Samples were applied to Whatman 3M paper at origin and electrophoresed at 7.5 volts/cm for 8 hr in sodium acetate buffer, pH 3.7. After drying, the paper was dipped in alkaline AgNO₃ to visualize the carbohydrates. Spot 1: 20 μg gluconic acid; spot 2: 20 μg gluconic acid; spot 3: 40 μg glucose. Sample B is a reaction mixture control using boiled enzyme, and spot 3 is equivalent to 40 μg glucose. Sample A is the reaction mixture after the glucose dehydrogenase reaction, showing remaining glucose (spot 3) and appearance of a new spot (5) which electrophoresed with gluconic acid (spot 2). Spot 4, an unknown, was present in the boiled control and the reaction mixture.
Lineweaver-Burk plots from which the $K_m$ values for the sugar substrates were derived are shown in Figure 4. In addition to glucose, the enzyme was equally active with the glucose derivative, 2-deoxyglucose. At 35°C, the $K_m$ for glucose with either pyridine nucleotide was 18 to 20 mm, whereas that for 2-deoxyglucose was slightly lower, 15 to 16 mm. At 22°C, the $K_m$ for both sugars was lower by about 50%; at this lower temperature, $K_m$ values were: glucose (NADP), 9 to 10 mm; glucose (NAD), 10 to 11 mm; and 2-deoxyglucose (NADP), 8 to 9 mm. Thus, the enzyme has an increased affinity for the sugar substrate at lower temperatures than 35°C.

The purified enzyme showed no activity with glucose-6-P and, in confirmation of our earlier report (8), no activity with D-xylose, D-fructose, or D-galactose at 35°C.

**Electron Acceptors.** Other electron acceptors at the following concentrations were inactive (showed less than 1% of the rate with pyridine nucleotide) when tested with the purified enzyme: dichlorophenol indophenol, 40 μM; ferricyanide, 440 μM; nitro blue tetrazolium, 60 μM; O$_2$, 1 mm; mammalian Cyt c, 25 μM; Cyt c$_{558}$ (recently described from strain MAC), about 25 μM; FMN, 1 to 5 μM; and FAD, 5 to 20 μM. The enzyme appeared restricted to use of pyridine nucleotides alone.

**Effectors of the Reaction.** The following carbohydrate intermediates were tested and showed no effect on the enzyme-catalyzed reaction: glucose-6-P (10 mm), gluconate (10 mm), gluconate-6-P (10 mm), and glycerate-3-P (20 mm). ADP at 2 to 5 mm did not stimulate or inhibit the reaction, nor did it lower the $K_m$ for glucose. ATP at 10 mm and EDTA at 5 mm pH 8 did not inhibit the reaction.

Mg$^{2+}$ at 10 mm was required for maximum activity, but the requirement was not absolute. Without added Mg$^{2+}$, the reaction with purified enzyme proceeded at about 70% of the Mg$^{2+}$-supported rate. Mn$^{2+}$ at 5 mm produced 50% inhibition whether Mg$^{2+}$ was present or not. This inhibition by Mn$^{2+}$ did not appear to be pH-related. Ca$^{2+}$ had no effect on enzyme activity, alone or with Mn$^{2+}$.

Inhibition of enzyme activity occurred with the following: KCN, 50% at 10 mm; NADPH, 13% at 0.27 mm; and atabrine, 50% at 1 mm.

**DISCUSSION**

GPND from dark-grown strain MAC is typical of GPND from other bacteria (1, 3, 9) and animal liver (5, 10) with respect to its high $K_m$ for glucose and an alkaline pH optimum. Aside from these characteristics, glucose dehydrogenases exhibit considerable variability and strain MAC GPND follows this trend.

Other substrates, particularly D-xylose, are utilized by GPND from *Bacillus anitratum* (3) and from beef liver (10), in contrast to strain MAC GPND. The GPND from strain MAC is one of the few glucose dehydrogenases described which shows a lower $K_m$ for NADP than NAD, although enzymes from a number of other species utilize both pyridine nucleotides as electron acceptors, e.g. GPND from *Bacillus cereus* spores (1) and GPND from animal liver (5, 10), with NAD being reduced faster than NADP. Enzymes from some species are inhibited by glucose-6-P (1, 10) in contrast to GPND from strain MAC. Activation by metal ions, too, has generally not been observed.

![Graph](https://www.plantphysiol.org)  
*Fig. 4.* Lineweaver-Burk plot, showing effects of glucose on NADP-linked (○○) and NAD-linked (●●) glucose dehydrogenase activity, and effect of 2-deoxyglucose on NADP-linked glucose dehydrogenase activity (ΔΔΔΔ), at temperatures of 22°C and 35°C. S: mol/l glucose or deoxyglucose; v: μmol NADP or NAD reduced/min. Assays were performed as described in "Materials and Methods" using 5 μg enzyme for 35°C assays and 2 μg enzyme for 22°C assays.
GPND from B. cereus spores (1) is the only other soluble GPND studied besides that of strain MAC which requires a metal ion; and it is activated by Mn2+.

While this work was in progress, a brief report on the glucose dehydrogenase from Chlorogloeopsis (Chlorogloea) frischii grown under photoautotrophic conditions was presented by Pritchard et al. (7). Generally, the properties of the Chlorogloea enzyme were similar to those described for the enzyme from dark-grown strain MAC with the exception of the reported activity with other sugars such as galactose and the lack of Mg2+ stimulation. It is also apparent that the Chlorogloea enzyme has a 4- to 5-fold higher Km for NADP than the strain MAC enzyme.

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LITERATURE CITED