Nicotinamide Adenine Dinucleotide- and Nicotinamide Adenine Dinucleotide Phosphate-specific Glucose 6-Phosphate Dehydrogenases of *Acetobacter xylinum* and Their Role in the Regulation of the Pentose Cycle*

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**SUMMARY**

Extracts of *Acetobacter xylinum* cells grown on glucose or succinate, oxidized glucose 6-phosphate to 6-phosphogluconate in the presence of either NAD or NADP. NAD-linked glucose 6-phosphate dehydrogenase and the corresponding NADP enzyme were completely separated and partially purified by DEAE-cellulose chromatography. The NAD enzyme was optimally active at pH 5.6, and the NADP enzyme at pH 8.2. Each enzyme exhibited strict specificity for its respective coenzyme. The interaction of glucose 6-phosphate and the corresponding coenzyme was that of a typical two-substrate system. The reaction rates with both enzymes were hyperbolic functions of coenzyme and substrate concentrations. The $K_m$ values for glucose 6-phosphate and the corresponding coenzyme were 2.5 mM and 0.08 mM for the NAD enzyme and 1.64 mM and 0.04 mM for the NADP enzyme. Magnesium ions did not affect the activity of the enzymes. The NAD enzyme was competitively inhibited by NADH with respect to NAD ($K_i = 0.035$ mM) and was not affected by NADPH. The NADP enzyme was competitively inhibited by NADPH with respect to NADP ($K_i = 0.028$ mM) and was insensitive to NADH. ATP inhibited the activity of the NAD glucose 6-phosphate dehydrogenase. The inhibition, which followed classical kinetic patterns, was competitive with respect to glucose 6-phosphate ($K_i = 1.3$ mM) and noncompetitive with respect to NAD. The NADP-linked enzyme was insensitive to ATP.

It is suggested that the NAD and NADP glucose 6-phosphate dehydrogenases have distinctly different functional roles. The possible relationship between the properties of the two enzymes and regulatory mechanisms in the control of hexose metabolism in *A. xylinum* is discussed.

**METHODS AND MATERIALS**

Cells—The strain of *A. xylinum* was the same as that employed in earlier investigations (5, 8). The culture media, prepared with glass-distilled water, were as follows: substrate (succinate of glucose), 2%; yeast extract (Difco), 0.5%; Bacto peptone (Difco), 0.5%; and monopotassium phosphate, 0.3%. The succinate medium was adjusted to pH 4.0 with NaOH and the glucose medium to pH 5.0 with HCl. Cells were grown and harvested after 24 hours as described previously (9).

Preparation of Extracts—Cells suspended in 5 mM Tris-H$_2$SO$_4$ buffer, pH 7.4, were treated for 15 min in a Raytheon model DF1 magnetorestrictive oscillator at 200 watts and 10 kc per s. The sonic extract was then centrifuged in the cold at 8000 × g for 15 min and the precipitate was discarded. The supernatant fluid ("crude extract"), which usually contained between 10 and 20 mg of protein per ml, was used for enzyme assays.

Enzymatic Assays—Enzyme assays involving the measurement of absorbance changes were performed at room temperature (about 22°C) in a Zeiss spectrophotometer or a Gilford model...
2000 recording spectrophotometer with 10-mm light path quartz cells. Changes in absorbance in the presence of NADH or NADPH were measured in cells of 5-mm light path.

Glucose 6-phosphate dehydrogenase activity was measured by following the increase in absorption at 340 nm resulting from the glucose 6-phosphate-dependent reduction of NAD or NADP. The standard assay system for the NAD-linked glucose 6-phosphate dehydrogenase contained 100 mM Tris–maleate buffer, pH 6.9, 0.5 mM NAD, enzyme, 15 mM glucose 6-phosphate, and water to a final volume of 1 ml. The standard assay system for the NADP-linked glucose 6-phosphate dehydrogenase contained 100 mM Tris-HSO₄ buffer, pH 8.2, 0.5 mM NADP, enzyme, 15 mM glucose 6-phosphate, and water to a final volume of 1 ml. The reactions were started by addition of glucose 6-phosphate. In each case the reaction rate was proportional to enzyme concentration.

6-Phosphogluconate dehydrogenase, phosphohexoisomerase, and phosphoglucomutase were assayed as described by Gromet, Schramm, and Hestrin (1). All enzyme units are expressed as the amount of enzyme catalyzing the transformation of 1 μmole of substrate per min under the conditions described. Specific activity is expressed in units per mg of protein.

Analytical Methods—Glucose 6-phosphate and NADP were determined with yeast glucose 6-phosphate dehydrogenase (10). NAD was determined with alcohol dehydrogenase, and NADH and NADPH with lactate dehydrogenase and glutamate dehydrogenase (11). 6-Phosphogluconate was assayed with yeast 6-phosphogluconate dehydrogenase and NADP. Protein was estimated by the method of Lowry et al. using a crystalline bovine serum albumin standard (12).

Chemicals—Nucleotides, sugar phosphates, pyruvate, oxaloacetate, 6-phosphogluconate, 2-phosphoglycerate, 2,3-diphosphoglycerate, and most of the enzymes were purchased from Boehringer and Soehne, Mannheim, Germany. Glyceraldehyde 3-phosphate, phosphoenolpyruvate, and p-hydroxymercuribenzoate were obtained from Sigma Chemical Co., St. Louis, Mo.

RESULTS

Glucose 6-Phosphate-dependent Reduction of NAD and NADP—Cell-free extracts of A. xylinum catalyzed the reduction of both NAD and NADP in the presence of glucose 6-phosphate. Under optimal assay conditions (see "Methods and Materials"), the two coenzymes were reduced at approximately the same rate (0.02 to 0.025 μmole per min per mg of protein). The specific activity of the glucose 6-phosphate-dependent reduction of NAD and NADP was virtually the same in extracts obtained from cells grown on either glucose or succinate as carbon source.

Separation and Purification of NAD- and NADP-linked Glucose 6-Phosphate Dehydrogenases—The steps reported below were carried out at 0 °C. A crude extract of succinate-grown cells was centrifuged in a Beckman model L-2 ultracentrifuge at 150,000 × g for 60 min and the sediment was discarded. The supernatant was applied to a column of DEAE-cellulose which had been previously equilibrated with 5 mM Tris-HSO₄ buffer, pH 7.4. The column was eluted with a 200 ml linear gradient of 0.075 M KC1 to 0.25 M KC1 dissolved in the same buffer. Fractions (7 ml) were collected at a flow rate of 18 ml per hour and were assayed for glucose 6-phosphate dehydrogenase activity with NAD (O) or NADP (X) as described under "Methods and Materials." The numbers at the top of the figure indicate the concentration of KC1 at the peak positions.

FIG. 1. Separation of NAD-linked glucose 6-phosphate dehydrogenase and NADP-linked glucose 6-phosphate dehydrogenase by column chromatography on DEAE-cellulose. A crude extract of succinate-grown cells was centrifuged at 150,000 × g for 60 min and the sediment was discarded. The supernatant (15 ml containing 136 mg of protein) was applied to a column (1.2 × 14 cm) of DEAE-cellulose (Whatman DE-52), which had been previously equilibrated with 5 mM Tris-HSO₄ buffer, pH 7.4. The column was eluted with a 200 ml linear gradient of 0.075 M KC1 to 0.25 M KC1 dissolved in the same buffer. Fractions (7 ml) were collected at a flow rate of 18 ml per hour and were assayed for glucose 6-phosphate dehydrogenase activity with NAD (O) or NADP (X) as described under "Methods and Materials." The numbers at the top of the figure indicate the concentration of KC1 at the peak positions.

6-Phosphogluconate dehydrogenase activity and of four tubes containing the NADP-linked activity were pooled separately and the enzymes were precipitated by addition of ammonium sulfate to 50% saturation. The precipitates were dissolved in 50 mM Tris-HSO₄ buffer (pH 7.4) and dialyzed overnight against this buffer with several changes. "Fraction I" containing the NAD-glucose 6-phosphate dehydrogenase had a specific activity of 0.5, representing a 23-fold purification over the crude extract with 65% recovery of the total activity. "Fraction II" containing the NADP-linked enzyme had a specific activity of 0.51 with 48% recovery of the total activity and 20-fold purification over the crude extract. Both fractions contained no detectable 6-phosphogluconate dehydrogenase, phosphohexoisomerase, or phosphoglucomutase activity. When stored in the frozen state these fractions retained most of their activity over a period of 3 to 4 weeks. They were used for the studies described in the following sections.

Stoichiometry of Reactions—The stoichiometry of glucose 6-phosphate dehydrogenation as catalyzed by the partially purified enzymes is shown in Table I. With Fraction I the utilization of equimolar amounts of glucose 6-phosphate and NAD was accompanied by the production of equivalent amounts of 6-phosphogluconate and NADH. With Fraction II equivalent amounts of glucose 6-phosphate and NADP were utilized and 6-phosphogluconate and NADPH were formed. When 6-phosphogluconate was incubated with either NADH and Fraction I or NADPH and Fraction II, no oxidation of the reduced coenzymes could be demonstrated. This suggests that the primary product of glucose 6-phosphate oxidation is not 6-phosphogluconic acid but rather the 6-phosphogluconolactone which is subsequently hydrolyzed to the free acid.

pH Dependence—The pH-activity profiles of the two enzymes are shown in Fig. 2. The NAD-linked dehydrogenase possesses a sharp activity maximum at pH 5.6. Little or no activity was
observed above pH 6.2. The NADP-linked enzyme possesses maximal activity at pH 8.0 to 8.2. Activity decreased gradually at lower pH values to 33% of maximal activity at pH 5.6.

Kinetic Constants for Substrates—Double reciprocal plots of velocity against coenzyme concentrations at different levels of glucose 6-phosphate are shown in Fig. 3 for the NAD enzyme and in Fig. 4 for the NADP enzyme. The $K_m$ values for glucose 6-phosphate and the corresponding coenzyme calculated from these plots are 2.5 mM and 0.08 mM for the NAD-linked enzyme and 1.64 mM and 0.04 mM for the NADP-linked enzyme.

Effect of Magnesium—The activity of both enzymes was not affected by the addition to reaction mixtures of 10 mM magnesium ions. Furthermore, activity was not modified by the presence of 10 mM ethylenediaminetetraacetate.

Coenzyme and Substrate Specificity—The glucose 6-phosphate dehydrogenase of Fraction I showed strict NAD specificity, while that of Fraction II was highly specific for NADP. Under standard assay conditions for the former enzyme no activity was observed when NAD was substituted by 2 mM NADP. Similarly, no activity could be detected with 2 mM NAD in the standard assay for the NADP enzyme of Fraction II.

Both enzymes did not catalyze the reduction of their respected

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**Table 1**

Stoichiometry of reactions catalyzed by glucose 6-phosphate dehydrogenases

<table>
<thead>
<tr>
<th>System</th>
<th>Glucose 6-phosphate</th>
<th>NAD</th>
<th>NADP</th>
<th>6-Phos-</th>
<th>NADH</th>
<th>NADPH</th>
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<tr>
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<td>-0.46</td>
<td></td>
<td>+0.44</td>
<td>+0.41</td>
<td></td>
</tr>
<tr>
<td>II</td>
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<td></td>
<td>-0.63</td>
<td>+0.67</td>
<td>+0.65</td>
<td></td>
</tr>
</tbody>
</table>

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Fig. 2. Effect of pH on the activity of NAD- and NADP-linked glucose 6-phosphate dehydrogenases. NAD- and NADP-linked glucose 6-phosphate dehydrogenase assays were as described in text, but for 0.1 M buffer of the desired pH. The following buffers were used: sodium acetate-acetic acid at pH 4.6 to 5.2, Tris-maleate at pH 5.2 to 8.4, Tris-HSO₄ at pH 7.4 to 8.8, and glycine-NaOH at pH 8.6 to 9.2. An amount of enzyme with 4 to 10 milliunits of activity at the respective pH was used in each assay. Observed velocities are relative to that at the optimal pH. °, NAD enzymes; ×, NADP enzyme.

Fig. 3 (left). Double reciprocal plots of velocity versus NAD concentration at various fixed levels of glucose 6-phosphate for the NAD-linked glucose 6-phosphate dehydrogenase. The concentrations of glucose 6-phosphate used were: 15 mM (■); 2.4 mM (○); 1.0 mM (×). The amount of enzyme (Fraction I) used per assay was an equivalent of 40 milliunits. Velocities are expressed as micromoles of NADH formed per min per mg of protein.

Fig. 4 (right). Double reciprocal plots of velocity versus NADP concentration at various fixed levels of glucose 6-phosphate for the NADP-linked glucose 6-phosphate dehydrogenase. The concentrations of glucose 6-phosphate used were: 15 mM (■); 1.6 mM (○); 1.0 mM (×). The amount of enzyme (Fraction II) used per assay was equivalent of 35 milliunits. Velocities are expressed as micromoles of NADPH formed per min per mg of protein.
tive coenzyme in the presence of 2 to 10 mM of the following sugar phosphates: glucose 1-phosphate, fructose 6-phosphate, mannose 6-phosphate, ribose 5-phosphate, fructose 1-phosphate, fructose 1,6-diphosphate, and 6-phosphogluconate.

**Inhibition by Reduced Coenzymes**—The NAD-linked glucose 6-phosphate dehydrogenase is competitively inhibited by NADH with respect to NAD, while the corresponding NADP-linked enzyme is competitively inhibited by NADPH with respect to NADP (Figs. 5 and 6). The $K_i$ values calculated from these Lineweaver-Burk plots are 0.035 mM for NADH and 0.028 mM for NADPH. The inhibitions were noncompetitive with respect to glucose 6-phosphate (Figs. 7 and 8). Activity of the NAD-linked enzyme was not affected by NADP and NADPH at concentrations up to 0.5 mM. Similar concentrations of NAD and NADH had no effect on the activity of the NADP-linked enzyme.

**Effect of ATP**—The NAD-linked enzyme is inhibited by ATP. Inhibition curves at constant ATP and variable substrate concentrations are given in Fig. 9. The results show that ATP inhibition is linear competitive with respect to glucose 6-phosphate with a $K_i$ of 1.3 mM. When the kinetics of inhibition by 1.5 and 5 mM ATP were similarly studied as a function of NAD concentration (between 0.05 to 1.0 mM), and at a fixed concentration of glucose 6-phosphate (2.0 mM), the results indicated a noncompetitive relationship between ATP and NAD (Fig. 10). The extent of ATP inhibition was not affected by varying the pH between 4.5 to 6.3.

Other nucleotides were tested for their effect on the NAD-dependent enzyme. In standard reaction mixtures, in the presence of 1 mM glucose 6-phosphate, 5 mM ATP inhibited activity by 70%. Inhibition by 5 mM GTP, CTP, ITP, UTP, or ADP was 35 to 45%, and by 5 mM AMP only 5 to 10%.

![Fig. 5. Inhibition of the NAD-linked glucose 6-phosphate dehydrogenase by NADH with NAD as the variable substrate in the constant presence of 0.5 mM NAD. The concentrations of NADH used were: 0 (○); 0.07 mM (○); 0.14 mM (△). The amount of enzyme (Fraction I) used per assay was equivalent of 42 milliunits. Velocities are expressed as in Fig. 3.](image1.png)

![Fig. 6. Inhibition of the NADP-linked glucose 6-phosphate dehydrogenase by NADPH with NADP as the variable substrate in the constant presence of 12 mM glucose 6-phosphate. The concentrations of NADPH used were: 0 (○); 0.05 mM (○); 0.12 mM (△). The amount of enzyme (Fraction II) used per assay was equivalent of 35 milliunits. Velocities are expressed as in Fig. 4.](image2.png)

![Fig. 7. Inhibition of the NAD-linked glucose 6-phosphate dehydrogenase by NADH with glucose 6-phosphate as the variable substrate in the constant presence of 0.5 mM NAD. The concentrations of NADH used were: 0 (○); 0.09 mM (○); 0.18 mM (△). The amount of enzyme (Fraction I) used per assay was equivalent of 42 milliunits. Velocities are expressed as in Fig. 3.](image3.png)
Fig. 8. Inhibition of the NADP-linked glucose 6-phosphate dehydrogenase by NADPH with glucose 6-phosphate as the variable substrate in the constant presence of 0.5 mM NADP. The concentrations of NADPH used were: 0 (○); 0.05 mM (○); 0.18 mM (×). The concentration of glucose 6-phosphate was 2.0 mM and the amount of enzyme (Fraction II) used per assay was an equivalent of 36 milliunits. Velocities are expressed as in Fig. 4.

Fig. 9. Inhibition of NAD-linked glucose 6-phosphate dehydrogenase by ATP; double reciprocal plot of velocity versus glucose 6-phosphate concentration at various levels of ATP. The concentrations of ATP used were: 0 (○); 1 mM (○); 2.5 mM (×). The concentration of NAD was 0.5 mM and the amount of enzyme (Fraction I) used per assay was an equivalent of 36 milliunits. Velocities are expressed as in Fig. 3.

At concentrations up to 10 mM, ATP was found to be without effect on the activity of the NADP-dependent glucose 6-phosphate dehydrogenase. This was tested in reaction mixtures containing glucose 6-phosphate and NADP at concentrations close to their respective \( K_m \) values, between pH 8.2 and 6.0.

Inhibition by \( p \)-Hydroxymercuribenzoate—Both the NAD- and NADP-linked enzymes are inhibited by \( p \)-hydroxymercuribenzoate. At equal enzyme concentrations (on a weight basis), 50% inhibition was obtained at 3 \( \times 10^{-6} \) M and 4 \( \times 10^{-6} \) M \( p \)-hydroxymercuribenzoate for the NADP- and NAD-linked enzymes, respectively. Thus, the sensitivity of the two enzymes to the sulphydryl reagent differs by a factor of 13.

Effect of Various Metabolites—A number of compounds related to carbohydrate metabolism were found to be without effect on either the NAD- or the NADP-linked glucose 6-phosphate dehydrogenases. Assays were made at substrate and coenzyme concentrations close to their respective \( K_m \) values. The compounds, tested at concentrations up to 10 mM, included fructose 6-phosphate, glucose 1-phosphate, fructose 1-phosphate, fructose 1,6-diphosphate, ribose 5-phosphate, 6-phosphogluconate, glucose 2-phosphate, glycerol 3-phosphate, 2,3-diphosphoglycerate, glyceraldehyde 3-phosphate, oxaloacetate, pyruvate, phosphoenolpyruvate, citrate, isocitrate, phosphate, and pyrophosphate.

DISCUSSION

Most of the glucose 6-phosphate dehydrogenases which display dual pyridine nucleotide specificity consist of a single enzyme capable of reacting both with NAD and NADP (13-16). The separation of NAD-linked glucose 6-phosphate dehydrogenase from the corresponding NADP enzyme reported here, demonstrates that in \( A. \) \( xylinum \), these activities are associated with separate proteins. The NAD-linked glucose 6-phosphate dehydrogenase of \( A. \) \( xylinum \) is similar to that of yeast (17) and \( E. \) \( coli \) (18) with respect to coenzyme specificity and pH optimum. The NAD enzyme, on the other hand, is unique in its strict specificity for NAD and low optimum pH. The response of both enzymes to varying substrate and coenzyme concentrations corresponds essentially to the kinetics of two-substrate reactions observed for a number of other pyridine nucleotide dehydrogenases, and is consistent with a sequential mechanism (19-22).

In mammalian, yeast, and many microbial systems, the metabolic role assigned to the pentose phosphate cycle is mainly anabolic, in generating metabolites and reducing equivalents (NADPH) for biosynthesis (15, 20, 23-26). In \( A. \) \( xylinum \), however, in addition to its biosynthetic function, which includes the generation of the hexose phosphate precursor of cellulose, the pentose phosphate cycle is the major pathway for the oxidative dissimilation of carbohydrates. This is achieved either through cycling, or in conjunction with the citrate cycle at the triose phosphate or acetate level (3, 27). The occurrence in \( A. \) \( xylinum \) of two distinct glucose 6-phosphate dehydrogenases is thus compatible with the dual metabolic function of the pentose
phosphate cycle in this organism. Furthermore, in accordance with the hypothesis put forward in other cases in which distinct NAD- and NADP-linked dehydrogenases were found to catalyze the same reaction in the cell (28-32), it is proposed that the NAD-linked glucose 6-phosphate dehydrogenase of A. xylinum is involved mainly when the pentose-phosphate pathway is directed toward oxidation and energy generation, whereas the NADP-linked enzyme functions in an anabolic capacity. The observations that the NAD-specific enzyme is sensitive to inhibition by ATP, whereas the NADP-dependent dehydrogenase is not, are consistent with this suggestion. The ATP-linked control of the NAD glucose 6-phosphate dehydrogenase, acting as a negative feedback, may serve as a physiological mechanism by which A. xylinum can attain the most efficient pattern of hexose phosphate utilization and regulate NADII and NADPH formation to match the relative demands of these compounds under various physiological conditions.

Both glucose 6-phosphate dehydrogenase of A. xylinum appear to be constitutive, being found at equivalent levels in cells grown on glucose or succinate. Growth on glucose requires the functioning of the NAD glucose 6-phosphate dehydrogenase as well as the NADP-linked dehydrogenase. During growth on succinate, on the other hand, when hexose phosphate is produced via gluconeogenesis (8, 33), an uncontrolled oxidation of glucose 6-phosphate would restrict the supply of the hexose phosphate required for cellulose biosynthesis, which is essential for the growth of A. xylinum in a static medium (34). Such a situation could possibly be avoided when the cellular level of ATP, generated by the oxidation of succinate in the citrate cycle (35, 36), is high enough to inhibit the NAD-linked glucose 6-phosphate dehydrogenase.

An additional factor which could provide A. xylinum with a mechanism for regulating the flow of hexose phosphate into the pentose cycle is the low affinity of its glucose 6-phosphate dehydrogenase for variously labeled pyruvates which is not compatible with the operation of a pentose cycle on gluconeogenic hexose phosphate (5).

As regards its pH optimum and low affinity for hexose phosphate, it is noteworthy that the NAD-linked glucose 6-phosphate dehydrogenase of A. xylinum resembles the fructose 6-phosphate phosphoketolase of this organism which is likewise assumed to be involved in the energy metabolism of carbohydrates (27). The pH optimum for these two enzymes, pH 5.6, is in the pH range of maximum sugar oxidation and polymerization to cellulose by whole cells and is the same as that measured in unbuffered broken cell suspensions of this organism (38, 39). At this pH the activity of the NADP-linked glucose 6-phosphate dehydrogenase would probably still be enough to fulfill its anabolic function in the cell.

The results of in vivo experiments on the pattern of hexose phosphate utilization by A. xylinum under various physiological conditions, which are in accord with the mechanisms proposed here will be reported elsewhere.

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