Multiple Enzyme Forms of Glyceraldehyde-3-phosphate Dehydrogenase in 
*Pseudomonas aeruginosa* PAO

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(Received 27 April 1987; revised 24 June 1987)

Both NAD- and NADP-dependent glyceraldehyde-3-phosphate dehydrogenase (G3PDH) (EC 1.2.1.12) activities were detected in glucose-grown cells of *Pseudomonas aeruginosa* strain PAO. After growth on gluconeogenic substrates such as citrate, the activity of the NAD-G3PDH was reduced severalfold in contrast to little change for the NADP-G3PDH. The two G3PDH activities could be separated by ammonium sulphate fractionation. PAGE revealed the presence of two G3PDH isoenzymes of 140 (NADP-specific) and 315 (NAD-specific) kDa. Slight differences were observed in the thermostabilities and pH optima of the two enzymes whereas the regulation of their activities by various compounds varied strongly. The NADP-G3PDH enzyme was activated by ATP, reduced NAD, and fructose 6-phosphate. It was inhibited by fructose 1,6-diphosphate and 6-phosphogluconate. The NAD-G3PDH enzyme was inhibited by ATP, reduced NAD, and 6-phosphogluconate; it was slightly activated by reduced NADP. The possible roles of these isoenzymes in the control of hexose catabolism and gluconeogenesis in *P. aeruginosa* are discussed.

INTRODUCTION

*Pseudomonas aeruginosa* and *Pseudomonas* species in general exhibit exceptional nutritional versatility and induce many catabolic enzymes. The regulatory mechanisms governing their catabolic pathways have drawn increasing attention (Berka & Lessie, 1984; Clarke, 1972; Mal'tseva & Golovleva, 1985). In contrast, the enzymes involved in the pathways of central metabolism are generally synthesized constitutively and regulated at the level of enzyme activity. Various studies have revealed the presence of multiple enzyme forms of glucose-6-phosphate dehydrogenase (Ben-Bassat & Goldberg, 1980; Lessie & Neidhardt, 1967; Lessie & Vander Wyk, 1972; Maurer et al., 1982) and 6-phosphogluconate dehydrogenase (Lee & Lessie, 1974; Lessie & Vander Wyk, 1972) in *Pseudomonas*. It has been proposed that multiple enzyme forms are important in the regulation of the relative rate of reduction of NAD and NADP and thereby regulate the energy-yielding and biosynthetic reactions in the cell. This is possible when these enzymes are controlled by separate mechanisms.

This study was initiated to identify and characterize possible multiple enzyme forms of glyceraldehyde-3-phosphate dehydrogenase (G3PDH) (EC 1.2.1.12) in *P. aeruginosa* and to relate their physiological role to carbohydrate catabolism in *Pseudomonas* species.

METHODS

Micro-organism. *Pseudomonas aeruginosa* strain PAO was originally obtained from B. W. Holloway (Monash University, Clayton, Victoria, Australia). Stock cultures were maintained at 4 °C on nutrient agar plates and transferred monthly. Cultures were permanently maintained in 5% (v/v) glycerol/1% (w/v) peptone (Difco).

Preparation of cell-free extract. Cultures were grown in autoclaved liquid minimal salts medium (Blevins et al., 1975) supplemented with the appropriate filter-sterilized carbon source at 20 mM final concentration. A 1:20

Abbreviations: G3PDH, glyceraldehyde-3-phosphate dehydrogenase; G3P, glyceraldehyde 3-phosphate.

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dilution of an exponential-phase starter-culture was used to initiate growth. Cultures were grown in flasks with a 1:1 working volume in an incubator-shaker (New Brunswick Scientific) at 37 °C and 200 r.p.m. until the optical density reached 0.5 at 660 nm, as determined in a B & L Spectronic 20 colorimeter (Bausch & Lomb). Cells were harvested by centrifugation at 4 °C and 10000 g for 10 min. The pellets were washed with 0.1 M-Tris/HCl buffer pH 8.5 containing 10 mM-2-mercaptoethanol, resuspended in the same buffer at 20 °C concentration and ruptured at 4 °C with an Amino French pressure cell using a Wabash hydraulic press at 7600 lbf in² (0.052 MPa). The cell debris was removed by centrifugation at 33000 g and 4 °C for 15 min. The cell-free extract was assayed immediately for G3PDH activity.

Assays were also completed on a partially purified extract prepared from the crude cell-free extract. An equal volume of 2% (w/v) streptomycin sulphate was added at 4 °C for 15 min to remove nucleic acids. The extract was then centrifuged at 17000 g and 4 °C for 20 min. To the supernatant, ammonium sulphate was added to 80% saturation at 4 °C for 30 min. The extract was then centrifuged at 17500 g and 4 °C for 20 min. The pellet was resuspended in the same buffer and assayed immediately for G3PDH activity.

**Assay of G3PDH.** The assay procedure for G3PDH was a modification of the method of Tiwari & Campbell (1969). The assay mixture contained, in 1 ml, 100 mM-Tris/HCl, 6 mM-DL-glyceraldehyde 3-phosphate (G3P), 10 mM-cysteine, HCl, 17 mM-sodium arsenate, 20 mM-sodium fluoride, 0.25 mM-NAD or NADP, and 0-10 ml cell-free extract. Barium was removed from the G3P by treatment with Dowex 50W resin. The G3P and cysteine, HCl were neutralized immediately before use with 1 M-NaOH. The reaction mixture minus G3P was incubated at 30 °C for 1 min; the reaction was initiated by adding the G3P. The reduction of either NAD or NADP was followed at 340 nm with a Beckman Kintrac VII recording spectrophotometer. Protein concentrations were determined by the method of Lowry and specific activities were determined using the absorption coefficients for NAD and NADP respectively. Activities are expressed in international units (IU) and were determined from the linear range by duplicate assays of experiments run in triplicate. The data presented are representative of those observed throughout the study.

**Ammonium sulphate fractionation.** The nucleic-acid-free supernatant obtained after 2% streptomycin sulphate treatment (see above) was used for the ammonium sulphate fractionations. Highly purified ammonium sulphate was added to give successive increases of 5% saturation up to 70% saturation. The precipitates from each fractionation were resuspended in a small volume of buffer and assayed for NAD- and NADP-specific G3PDH activity.

**Effects of physical and chemical factors on the activity of G3PDH.** (i) **Effect of carbon source.** *P. aeruginosa* PAO was grown in minimal salts medium containing a 20 mM final concentration of one of the following carbon sources: glucose, gluconate, citrate, lactate, acetate or succinate, as stated previously. Cell-free extracts were prepared and assayed immediately for NAD- and NADP-specific G3PDH activity.

(ii) **Thermostability.** The thermal stability of the respective pyridine-nucleotide-specific reactions was determined with cell-free extracts of glucose-grown *P. aeruginosa* PAO. Samples (1-0 ml) were placed in small tubes and incubated for 5 min at various temperatures from 10 to 60 °C at 5 °C intervals with an equilibration period of 1 min at the temperature being investigated. After cooling on ice, the remaining activities were determined at 30 °C (see above).

(iii) **Effect of pH.** The effect of pH on NADP-specific G3PDH activity was determined with cell-free extracts of glucose-grown *P. aeruginosa* PAO. Assays were made at intervals from pH 4.0 to 12.0 in 0.1 M-Tris/HCl buffer pH 4.0-10.0 and 0.1 M-glycine buffer pH 10.0-12.0. Cell-free extract (0.1 ml) and 0.5 ml buffer were used in a total volume of 1.0 ml.

**Relative activities of NAD- and NADP-specific G3PDH during diauxic growth.** A diauxic growth experiment was designed to investigate the relative activities of NAD- and NADP-specific enzyme forms. *P. aeruginosa* PAO was grown in 25 l aerated minimal salts medium containing 2 mM-succinate and 10 mM-glucose at 37 °C; the culture was inoculated with exponential-phase succinate-grown cells. Samples (1 l) were removed at various times after inoculation, harvested and washed, and cell-free extracts were prepared and assayed immediately for NAD- and NADP-specific activity.

**Effect of modulators on G3PDH activity.** The effect of modulators on the NAD- and NADP-specific G3PDH activity was determined with cell-free extracts of glucose-grown cells. The assay mixture was that listed previously, except that 0.1 ml buffer was replaced with 0.1 ml 0.01 M of the desired modulator. In the case of reduced pyridine nucleotides, 10 μl of either 12.8 mM reduced NAD or 11.0 mM reduced NADP was added to the assay mixture.

**Resolution of multiple enzyme forms of G3PDH by PAGE.** A modification of the method of Davis (1964) was used for PAGE. Gels consisting of 7.5% (w/v) polyacrylamide were prepared by mixing polyacrylamide and ammonium persulphate. N,N,N',N'-tetramethylethylenediamine (TEMED) was added as a polymerization catalyst. Gels were placed in a Buchler PAGE chamber and 0.1 ml samples of a cell-free extract in 10% (v/v) glycerin density medium containing 0.001% bromophenol blue were applied. A 0.1 M-Tris/HCl pH 8.5 buffer was placed in both the upper and lower (anodic) chambers. Current was applied until the tracking dye reached the bottom of the gels. The gels were stained specifically for NAD- and NADP-specific G3PDH activity with
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Table 1. Effect of carbon source on NAD- and NADP-specific G3PDH activities

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Specific activity (IU)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NAD</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.116</td>
</tr>
<tr>
<td>Gluconate</td>
<td>0.102</td>
</tr>
<tr>
<td>Citrate</td>
<td>0.029</td>
</tr>
<tr>
<td>Lactate</td>
<td>0.021</td>
</tr>
<tr>
<td>Acetate</td>
<td>0.013</td>
</tr>
<tr>
<td>Succinate</td>
<td>0.029</td>
</tr>
</tbody>
</table>

phenazine methosulphate (0.5 mg ml⁻¹) and 2,3,5-triphenyltetrazolium chloride (3.0 mg ml⁻¹) at 30 °C in a double-strength concentration of the assay mixture listed previously. Bands of G3PDH were observed after incubating gels at 30 °C for 90 min, after which time the reaction was stopped with the addition of glacial acetic acid (0.5 ml). Molecular masses were estimated by comparison of the migration distance of the NAD and NADP enzyme forms to that of equine heart myoglobin (16.89 kDa), rabbit muscle G3PDH (140 kDa) and Escherichia coli β-galactosidase (521 kDa) by the method of Hedrick & Smith (1969).

Chemicals and reagents. All chemicals and reagents were from Sigma except those used for the preparation of polyacrylamide gels, which were obtained from Bio-Rad.

RESULTS AND DISCUSSION

Ammonium sulphate fractionation

Maximal activity of NAD- and NADP-specific G3PDH activities occurred at 70% (NAD) and 55% (NADP) of ammonium sulphate saturation respectively. This provided initial evidence that at least two forms of G3PDH, one specific for NAD and one specific for NADP, were present.

Effects of physical and chemical factors on the activity of G3PDH

Effect of carbon source. Initially, various glycolytic and gluconeogenic substrates were used as the sole carbon source. The effects of these carbon sources on NAD- and NADP-specific G3PDH activities (Table 1) indicate four to ninefold less NAD-specific activity on gluconeogenic substrate. Similar ranges of NAD- and NADP-specific activities were observed on glycolytic substrates. Repression of both NAD- and NADP-specific activities of glycolytic enzymes after growth on gluconeogenic carbon sources has been shown previously by O'Brien (1975) and Tiwari & Campbell (1969).

In order to follow the relative induction and repression of the NAD- and NADP-specific forms of G3PDH, a diauxic growth experiment using succinate and glucose was implemented; Tiwari & Campbell (1969) have shown that P. aeruginosa uses succinate preferentially over glucose. The data (Fig. 1) show ratios of activities for NADP-G3PDH:NAD-G3PDH, and thereby increases and decreases of the two activity forms relative to each other. As succinate was depleted from the medium, the ratio of NAD-specific to NADP-specific activity increased dramatically. This most probably reflects the relative importance of the NAD-specific form in glucose catabolism, and may lead to increased production of NADH.

Thermostability. Both the NAD- and the NADP-specific forms of G3PDH were stable when incubated at 15 °C for 5 min. Total loss of activity was observed at 55 °C for NAD-specific G3PDH and at 50 °C for NADP-specific G3PDH. The NAD-G3PDH remained stable over a broader range of temperatures than the NADP-dependent enzyme. This was expected because the NAD form had been shown to be stable when stored in 75% glycerol containing 1 mM-EDTA at 4 °C. The NADP form, on the other hand, lost activity within 6 h regardless of the storage conditions; this may explain why multiple enzyme forms of G3PDH have not been shown previously in P. aeruginosa.
Effects of pH. The in vitro pH optima were 9.25 for the NAD-specific activity and 8.5 for the NADP-specific activity. The activity of both pyridine-nucleotide-specific enzymes decreased rapidly on either side of their respective pH optimum. The pH optima are in the same range of pH as those determined for G3PDH in other species (Amelunxen, 1967; Crow & Wittenberger, 1976).

Effect of modulators. The effects of selected modulators on G3PDH activity were studied to indicate possible control mechanisms involved. Previous studies have shown that multiple enzyme forms of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase were controlled by purine nucleotides and reduced NADP (Ben-Bassat & Goldberg, 1980; Lien et al., 1979; Maurer et al., 1982), and that substrate-dependent cooperative conformational changes may be involved (Demark et al., 1983). If multiple forms of G3PDH are to function

Table 2. Effect of modulators on NAD- and NADP-specific G3PDH activities

<table>
<thead>
<tr>
<th>Modulator</th>
<th>NAD</th>
<th>NADP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>Fructose 6-phosphate</td>
<td>99.6</td>
<td>220.6</td>
</tr>
<tr>
<td>Fructose 1,6-diphosphate</td>
<td>100.6</td>
<td>0.0</td>
</tr>
<tr>
<td>6-Phosphogluconate</td>
<td>73.4</td>
<td>78.8</td>
</tr>
<tr>
<td>AMP</td>
<td>87.3</td>
<td>112.1</td>
</tr>
<tr>
<td>ATP</td>
<td>71.4</td>
<td>139.4</td>
</tr>
<tr>
<td>Reduced NAD</td>
<td>89.0</td>
<td>480.0</td>
</tr>
<tr>
<td>Reduced NADP</td>
<td>110.7</td>
<td>105.8</td>
</tr>
</tbody>
</table>
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Fig. 2. Resolution of two forms of G3PDH by PAGE. G3PDH was detected by incubating the gels in double strength assay mixture at 30 °C for 90 min with NADP as cofactor (1), with NAD and NADP as cofactors (2), or with NAD as cofactor (3). A, NADP-dependent activity; B, NAD-specific activity.

similarly at an important regulatory point in the glycolytic scheme of P. aeruginosa PAO, they may be under a similar type of control. Table 2 shows the relative effects of various modulators on the enzyme activity. ATP was an inhibitor of the NAD-specific form and an activator of the NADP-specific G3PDH. The NAD-specific inhibition may involve feedback inhibition when sufficient ATP has accumulated and reduced NAD is no longer required to produce additional ATP. Simultaneously, ATP may activate the NADP-specific enzyme so that elevated levels of reduced NADP may be formed for reductive biosynthesis. AMP had much less effect on activities than ATP. Reduced NAD appeared to act as a modulator and may be the primary activator of the NADP-specific G3PDH. For example, in the presence of reduced NAD, NADP-specific activity increased by 480% and therefore may be important in the control of reductive biosynthesis. Reduced NAD slightly inhibited the NAD-specific form and reduced NADP had a slight activating effect upon both forms of G3PDH. Fructose 6-phosphate was another activator of the NADP-specific enzyme. Because fructose 6-phosphate is apparently not converted to fructose 1,6-diphosphate in P. aeruginosa (Blevins et al., 1975), high levels of fructose 6-phosphate may act as a signal that biosynthesis is favoured. Fructose 1,6-diphosphate acted as a powerful negative effector of the NADP-specific enzyme. 6-Phosphogluconate inhibited activity of both NAD- and NADP-specific G3PDH with similar effectiveness.

Resolution of multiple forms of G3PDH by PAGE

Resolution of NAD- and NADP-specific G3PDH was achieved by PAGE (Fig. 2). The NAD-specific form migrated ahead of the NADP-specific form. The staining assay contained only NADP for gel 1, both NAD and NADP for gel 2, and only NAD for gel 3. The estimated molecular masses of the two G3PDH enzymes based upon relative electrophoretic mobilities were 315 and 140 kDa for the NAD- and NADP-specific enzymes respectively.
Conclusions

Two distinct forms of G3PDH dependent upon either NAD or NADP, resolved by separation in PAGE, were also distinct in other important areas, including ammonium sulphate fractionation, thermostability, pH, response to potential modulators, and relative levels during diauxic growth on gluconeogenic and glycolytic carbon sources. The NAD-specific G3PDH appears to mediate catabolic reactions, while the NADP-specific G3PDH appears to mediate gluconeogenic reactions.

This study was partially supported by Auburn University Grant-in-Aid 73-107. The authors appreciate the excellent technical assistance of Brenda Sconyers and the advice of Drs T. W. Feary and P. V. Phibbs, Jr, during portions of this study.

REFERENCES


