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Purification and characterization of glycerol-3-phosphate dehydrogenase of *Saccharomyces cerevisiae*

Jacobus Albertyn, André van Tonder and Bernard A. Prior

Department of Microbiology and Biochemistry, University of the Orange Free State, PO Box 339, Bloemfontein 9300, South Africa

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The NAD-dependent glycerol-3-phosphate dehydrogenase (glycerol-3-phosphate:NAD$^+$ oxidoreductase; EC 1.1.1.8; G3P DHG) was purified 178-fold to homogeneity from *Saccharomyces cerevisiae* strain H44-3D by affinity- and ion-exchange chromatography. SDS-PAGE indicated that the enzyme had a molecular mass of approximately 42,000 (± 1,000) whereas a molecular mass of 68,000 was observed using gel filtration, implying that the enzyme may exist as a dimer. The pH optimum for the reduction of dihydroxyacetone phosphate (DHAP) was 7.6 and the enzyme had a pI of 7.4. NADPH will not substitute for NADH as coenzyme in the reduction of DHAP. The oxidation of glycerol-3-phosphate (G3P) occurs at 3% of the rate of DHAP reduction at pH 7.0. Apparent K_m values obtained were 0.023 and 0.54 mM for NADH and DHAP, respectively. NAD, fructose-1,6-bisphosphate (FBP), ATP and ADP inhibited G3P DHG activity. K_i values obtained for NAD with NADH as variable substrate and FBP with DHAP as variable substrate were 0.93 and 4.8 mM, respectively.

Yeast; Purification; Glycerol-3-phosphate dehydrogenase; Molecular mass

1. INTRODUCTION

Glycerol-3-phosphate (G3P) dehydrogenase (DHG) has been isolated from the cytoplasm of various microorganisms, including yeasts [1–4], algae [5–7] and bacteria [8–10]. The cytoplasmic G3P DHG of *Saccharomyces cerevisiae* catalyzes the reduction of DHAP to G3P, which is followed by dephosphorylation of G3P to glycerol by a phosphatase. Glycerol is an important product of glycolysis in *S. cerevisiae* and higher amounts are also produced as a compatible solute when yeast is exposed to osmotic stress. G3P DHG levels in *S. cerevisiae* are subject to glucose repression [11] and are also osmoregulated [12,13]. G3P DHG in *S. cerevisiae* was previously purified and characterized by Merkel et al. [2] and Chen et al. [3]. Discrepancies, however, exist between their data. The purpose of this investigation was to purify and characterize the G3P DHG from *S. cerevisiae* grown under osmotic stress. The effect of major intracellular solutes on the enzyme activity and kinetic constants were determined.

2. MATERIALS AND METHODS

2.1. *Yeast strain and cultivation*

S. cerevisiae strain H44-3D was cultured in YPD medium, containing 20 g · l^{-1} glucose, 20 g · l^{-1} peptone, 10 g · l^{-1} yeast extract and 0.05 g · l^{-1} ampicillin, in a fermentor (Virtis; Gardiner, New York) with an 8 l working volume. The water activity (a_w) of the medium was adjusted to 0.97 with NaCl (0.9 molal) [14]. The pH was set to 4 but not controlled during fermentation. The fermentor culture was incubated at 30°C with an air flow of 7.5 l · min^{-1}. Cells were harvested in mid-exponential phase by centrifugation at 8,000 × *g* and washed twice with isotonic solution at 4°C.

Correspondence address: J. Albertyn, Department of Microbiology and Biochemistry, University of the Orange Free State, PO Box 339, Bloemfontein 9300, South Africa. Fax: (27) (51) 474152.

2.2. *Purification of glycerol-3-phosphate dehydrogenase*

The cell extract was prepared by suspending 60 g yeast cells in 10 mM Tris-HCl buffer (pH 7.2) containing 10 mM EDTA, 2 mM 2-mercaptoethanol, 1 mM phenylmethanesulphonyl fluoride, 1 μM pepstatin and 0.1 mM antipain, and breaking the cells for 3 min in a Braun MSK Cell Homogenizer (Braun Melsungen AG, Germany). Cell debris was removed by centrifugation at 24,000 × *g* for 1 h and further manipulations were performed on the supernatant at 4°C. The sample was desalted and the buffer was changed by means of gel filtration (Sephadex G-25 column, 1.5 × 37 cm equilibrated with 10 mM Tris at pH 7.0). Active fractions were pooled and loaded batchwise onto an affinity column (reactive blue 2-Sepharose CL-6B column, Bio-Rad, 2.4 × 7.5 cm) and washed with 10 mM Tris (pH 7.0) (buffer A). The G3P DHG was eluted with buffer A containing 5 mM NADH, at a flow rate of 1 ml · h^{-1}. Active fractions were pooled and applied to a DEAE-Sephacel column (2.4 × 4 cm), equilibrated 0.1 M Tris-HCl, 2 mM 2-mercaptoethanol, pH 7.0 (buffer B). The column was washed with buffer B and the G3P DHG was eluted with a linear gradient of buffer B and 1 M Tris-HCl, 2 mM 2-mercaptoethanol, pH 7.0 (buffer C) at a flow rate of 1 ml · h^{-1}. The enzyme eluted at Tris concentrations between 285 and 560 mM. Active fractions were pooled and applied to a Sephadex G-100 column (1.6 × 47 cm) equilibrated with buffer C and eluted at a flow rate of 0.5 ml · h^{-1}. Active fractions were pooled, concentrated against polyethylene glycol (PEG) 2000 and used for kinetic studies. A summary of a representative enzyme purification is presented in Table I.

2.3. *Enzyme assays and determination of kinetic constants*

Enzyme assays and kinetic constants were determined spectrophotometrically. One unit of enzyme activity is defined as the rate of conversion of 1 μmol substrate or product per min, and specific activities are given as U · mg^{-1} protein. DHAP reduction [15] and G3P oxidation [4] by G3P DHG were assayed as described previously.

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Table I

Purification table of *Saccharomyces cerevisiae* glycerol-3-phosphate dehydrogenase

Purification step	Volume (ml)	Activity (U)	Protein (mg)	Specific activity ($U \cdot mg^{-1}$)	Yield (%)	Purification factor
Cell extract	28	3.3	1,389	0.23	100	1
Sephadex G25	19	147	359	0.41	46	1.8
Reactive blue	39	83	40.3	2.1	26	9
DEAE-Sephacel	10	49	2.4	20.3	15	88
Sephadex G100	6.3	40	1.43	41.1	14	178

Apparent affinity constant (K_m) values for NADH and DHAP were determined from double-reciprocal plots, and apparent inhibition constants (K_i) values from Dixon plots. Protein concentrations were determined as previously described [16].

2.4. *Molecular mass, pI and pH determinations*

The molecular mass of the native enzyme was determined by comparing its mobility on a TSKgel HW55F column with that of known standards [17]. Molecular mass under denaturing conditions was estimated by SDS-PAGE using standard proteins [18]. Isoelectric focusing (IEF) [19] and pH optimum determination [20] was performed as previously described.

2.5. *Western-immunoblot analysis*

Preparation of antibodies against purified G3P DHG [21] and immunoblotting of proteins separated on 12% polyacrylamide gels were carried out using standard procedures (Amersham Kit code RPN.23).

3. RESULTS

3.1. *Stability of the enzyme*

G3P DHG was initially eluted from the Sephadex G-100 column with buffer B. In this buffer, activity was quickly lost, and no activity remained after storage at −20 or −80°C in the presence of 50% PEG 2000, 5 mM NADH or 5 mM DHAP. However, when the enzyme was eluted from the Sephadex G-100 column with buffer C more than 90% activity remained after storage at 4°C for several weeks.

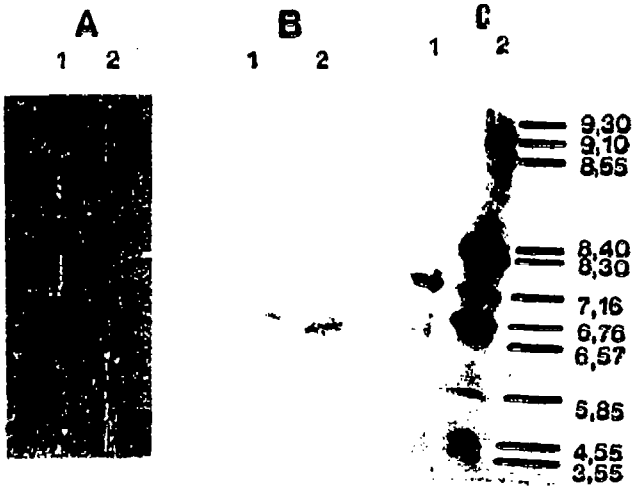

Fig. 1. (A) Silver-stained SDS-PAGE gel of G3P DHG (lane 2) purified from *S. cerevisiae*, with (lane 1) protein markers. (B) Western-immunoblot of (lane 1) crude extract from *S. cerevisiae* and (lane 2) purified G3P DHG. (C) IEF gel of purified G3P DHG (lane 1) and IEF standards (lane 2). 5 μg enzyme protein was applied in each case.

3.2. *Molecular mass, immunoblotting and isoelectric point*

The purified sample migrated as a single band on SDS-polyacrylamide gels with an apparent molecular mass of 42,000 (± 1,000; $n = 3$; Fig. 1). The molecular mass of the native enzyme as determined by gel filtration was estimated to be 68,000. This indicated that the undissociated enzyme may be a dimer. IEF revealed a pI of 7.4 (± 0.12; $n = 3$) for G3P DHG (Fig. 1). Immunoblotting confirmed the presence of a single protein under denaturing conditions in crude extracts and in the purified sample representing G3P DHG (Fig. 1).

3.3. *pH optimum, substrate specificity and coenzyme preference*

The maximum rate of DHAP reduction occurred at pH 7.6. Activity decreased rapidly below pH 7.0 and above pH 8.0, with activity decreasing more rapidly at the acidic pH. No activity could be detected at pH 5 and 11. NADPH could not substitute NADH as the coenzyme at pH values between 4.5 and 11 for the reduction of DHAP. G3P oxidation was 3% of the rate of DHAP reduction at pH 7.0, and no activity was observed when DHAP was replaced with dihydroxyacetone or glycerol as substrate.

3.4. *Kinetic studies*

The apparent K_m, V_{max} and K_i values are given in

Table II

Kinetic data of glycerol-3-phosphate dehydrogenase from *Saccharomyces cerevisiae*

Varied substrate	Inhibitor	K_m (mM)	V_{max} ($U \cdot mg^{-1}$)	K_i (mM)
NADH		0.023 (± 0.01)*	2.0 (± 0.13)	
DHAP		0.54 (± 0.03)	3.2 (± 0.5)	
NADH	NAD			0.93
DHAP	FBP			4.8

* Values represent the mean of three independent determinations with standard deviation in parentheses.

Table II. NAD (NADH as variable substrate) acted as a competitive inhibitor and FBP (DHAP as variable substrate) inhibited non-competitively. ATP at physiological concentrations of 1, 4 and 10 mM inhibited G3P DHG activity 83, 91 and 95%, respectively, whereas ADP at similar concentrations inhibited 36, 87 and 90%, respectively. No inhibition was observed with NADPH, G3P, acetaldehyde, glycerol and ethanol at concentrations of up to 1, 10, 10, 1,000 and 1,000 mM, respectively.

4. DISCUSSION

The properties of G3P DHG isolated from *S. cerevisiae* cultivated at 0.97 a_w (NaCl) differed in a number of instances from those described previously in *S. cerevisiae* [1–3], which may reflect differences between strains and cultivation conditions. The molecular mass of 42,000 obtained under denaturing conditions is the same as the mass reported for the G3P DHG from *S. cerevisiae* [3] and the salt-tolerant yeast, *Debaryomyces hansenii* [4]. Chen et al. [3] showed that their enzyme was a monomer, whereas the results of this study indicated that G3P DHG may be a dimer. In spite of previous studies on *S. cerevisiae* indicating G3P DHG to be a monomer [2,3], rabbit skeletal muscle G3P DHG possessed dimeric and monomeric active forms [22], and Nilsson and Adler [4] suggested that the dimeric form may also exist in *D. hansenii*.

The cofactor specificity of G3P DHG was limited to NADH, as previously observed in *S. cerevisiae* [2] and in *D. hansenii* [4], and NADPH could not replace NADH at any given pH. In contrast Nader et al. [1] reported that G3P DHG functioned at a very low rate with NADPH between pH 5 and 6 in *S. cerevisiae* (strain *carlsbergensis*). The considerably slower rate of G3P oxidation we observed compared to the rate of DHAP reduction indicates that the cytoplasmic G3P DHG functions primarily in glycerol biosynthesis but not in the utilization of glycerol. A mitochondrial G3P DHG oxidizes G3P to DHAP, and the inability of mutants of *S. cerevisiae* [11] and *D. hansenii* [23] lacking a mitochondrial G3P DHG supports further the assimilatory function of the cytoplasmic G3P DHG.

Metabolites found intracellularly differed in their ability to regulate the activity of G3P DHG. Glycerol, which is found in *S. cerevisiae* under non-stressed conditions at less than 2 mM and under osmotically stressed conditions up to 340 mM (unpublished data), failed to affect the enzyme activity, indicating that this solute is compatible with G3P DHG function. However, at physiological concentrations, ATP, ADP, NAD and FBP inhibited G3P DHG activity. In various yeasts, intracellular concentrations of ATP varied between 1 and 3.5 mM [24,25], NAD between 2 and 3 mM [26] and FBP between 3 and 10 mM [26]. At these concentrations, these metabolites will regulate the activity of G3P DHG in vivo. Furthermore, during osmotic stress the yeast cells shrink, which would initially increase metabolite concentrations. Oren-Shamir et al. [27] observed that the ATP concentration in the alga, *Dunaliella salina*, decreased during the recovery period from osmotic stress, and we have observed similar decreases in the yeast *Zygosaccharomyces rouxii* (unpublished data). If this metabolite decrease occurs in *S. cerevisiae*, the flux through G3P DHG would increase during recovery from osmotic shock.

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REFERENCES

[1] Nader, W., Betz, A. and Becker, J.-U. (1979) Biochim. Biophys. Acta 571, 177–185.
[2] Merkel, J.R., Straume, M., Sajer, S.A. and Hopfer, R.L. (1982) Anal. Biochem. 122, 180–185.
[3] Chen, S.-M., Trumbore, M.W., Osinchak, J.E. and Merkel, J.R. (1987) Prep. Biochem. 17, 435–446.
[4] Nilsson, L. and Adler, L. (1990) Biochim. Biophys. Acta 1034, 180–185.
[5] Haus, M. and Wegmann, K. (1984) Physiol. Plant. 60, 283–288.
[6] Haus, M. and Wegmann, K. (1984) Physiol. Plant. 60, 289–293.
[7] Klöck, G. and Kreuzberg, K. (1989) Biochim. Biophys. Acta 991, 347–352.
[8] Kistler, W.S. and Lin, E.C.C. (1972) J. Bacteriol. 112, 539–547.
[9] Schryvers, A., Lohmeier, E. and Weiner, J.H. (1978) J. Biol. Chem. 253, 783–788.
[10] Schryvers, A. and Weiner, J.H. (1981) J. Biol. Chem. 256, 9959–9965.
[11] Sprague, G.F. and Cronan, J.E. (1977) J. Bacteriol. 129, 1335–1342.
[12] Edgley, M. and Brown, A.D. (1983) J. Gen. Microbiol. 129, 3453–3463.
[13] Blomberg, A. and Adler, L. (1989) J. Bacteriol. 171, 1087–1092.
[14] Robinson, R.A. and Stokes, R.H. (1959) Electrolyte Solutions, 2nd edn., pp. 461, Scientific Publications, London.
[15] Gancedo, C., Gancedo, J.M. and Sols, A. (1968) Eur. J. Biochem. 5, 165–172.
[16] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[17] Andrews, P. (1965) Biochem. J. 96, 595–606.
[18] Laemmli, U.K. (1970) Nature 227, 680–685.
[19] Roberts, E.F., Dannelly, H.K., Molloy, P.J. and Reeves, H.C. (1987) Anal. Biochem. 167, 290–294.
[20] Todd, M.J. and Hausinger, R.P. (1987) J. Biol. Chem. 262, 5963–5967.
[21] Catty, D. and Raykundalia, C. (1988) in: Antibodies: A Practical Approach, vol. 1 (Catty, D. ed.) pp. 19–79, IRL Press, Oxford.
[22] Batke, J., Asboth, G., Lakatos, S., Schmitt, B. and Cohen, R. (1980) Eur. J. Biochem. 107, 389–394.
[23] Adler, L., Blomberg, A. and Nilsson, A. (1985) J. Bacteriol. 162, 300–306.
[24] Gustafsson, L. (1979) Arch. Microbiol. 120, 15–23.
[25] Larsson, C. and Gustafsson, L. (1987) Arch. Microbiol. 147, 358–363.
[26] Den Hollander, J.A., Ugurbil, K. and Shulman, R.G. (1985) Biochemistry 25, 212–219.
[27] Oren-Shamir, M., Pick, U. and Avron, M. (1990) Plant Physiol. 93, 403–408.