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PARTIAL PURIFICATION, SUBSTRATE SPECIFICITY AND REGULATION OF α-L-GLYCEROLPHOSPHATE DEHYDROGENASE FROM *SACCHAROMYCES CARLSBERGENSIS*

WERNER NADER, AUGUSTIN BETZ and JÖRN-ULLRICH BECKER

University of Bonn, Institute of Botany, D-5300 Bonn 1 (F.R.G.)

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Summary

α-L-Glycerolphosphate dehydrogenase (*sn*-glycerol-3-phosphate:NAD^+ 2-oxidoreductase, EC 1.1.1.8) from *Saccharomyces carlsbergensis* was purified 400-fold. The enzyme preparation is free of interfering activities, such as glyceraldehyde phosphate dehydrogenase, alcohol dehydrogenase, triose phosphate isomerase and glycerolphosphatase. At pH 7.0 it is specific for NADH (K_m = 0.027 mM with 0.8 mM dihydroxyacetone phosphate) and dihydroxyacetone phosphate (K_m = 0.2 mM with 0.2 mM NADH). Between pH 5.0 and 6.0 the enzyme functions with NADPH, but only at 7% of the rate with NADH. Various anions ($I^- > SO_4^{2-} > Br^- > Cl^-$) act as inhibitors competing with the substrate dihydroxyacetone phosphate. Inorganic phosphate (K_i = 0.1 mM), pyrophosphate and arsenate are strong inhibitors. The nucleotides ATP and ADP are also inhibitory, but their action seems to be of the same type as the general anion competition (K_i = 0.73 mM for ATP). The results are consistent with the notion that the enzyme may regulate the redox potential of the NAD^+/NADH couple during fermentation.

Introduction

A basic principle in fermentation is the need for balance of cellular reduction potentials. For every mol of NADH formed by yeast during oxidation of glyceraldehyde phosphate 1 mol of acetaldehyde is reduced to ethanol. When glycerol is the end product of fermentation (that is, through the so-called second 'Neuberg fermentation' [1]) the process is less energetically efficient. This occurs when acetaldehyde is withdrawn, for example, by addition of

sodium hydrogen sulfite, or if pyruvate is diverted for biosynthesis, as assumed by Lagunas [2]. Under these circumstances glycolysis would be limited by the lack of NAD^+, if it were not for the reoxidation of NADH by dihydroxyacetone phosphate and the subsequent dephosphorylation of α-L-glycerolphosphate by a specific phosphatase [3].

Even in the absence of added bisulfite, yeast forms glycerol in a constant ratio to ethanol production [4,5], which suggests that the reduction of dihydroxyacetone phosphate serves as a normal means of removing reducing equivalents. As up to 2% of glucose utilization occurs via the pentose phosphate pathway during fermentation by yeast [6], reducing $NADP^+$ in the process, it is clear that the NADPH formed thereby must be reoxidized. We considered that glycerol production might afford such a mechanism, if α-L-glycerolphosphate dehydrogenase (*sn*-glycerol-3-phosphate:NAD^+ 2-oxidoreductase, EC 1.1.1.8) was capable of using NADPH as electron donor.

In contrast to α-L-glycerolphosphate dehydrogenase from muscle [7,8], little is known about the yeast enzyme. Gancedo [3] estimated the enzyme activity in crude extracts of *Saccharomyces cerevisiae* and found it to be inhibited by solutions of high ionic strength. In order to explain the observations in crude extracts the enzyme was purified to a state free of enzyme activities which may interfere with the assays. In addition to examining the possibility that NADPH might act in the reduction of dihydroxyacetone phosphate, this communication characterizes the kinetic properties of yeast α-L-glycerolphosphate dehydrogenase and the effects of various ions on enzymatic activity.

Materials and Methods

α-L-Glycerolphosphate dehydrogenase was isolated from *Saccharomyces carlsbergensis*, ATCC Nr. 9080. The cells were grown in the medium described by Ghosh [9], using 12-l fermenter vessels at 28°C.

The activity of α-L-glycerolphosphate dehydrogenase was determined by following the rate of oxidation of NADH at 366 nm with an Eppendorf photometer equipped with a linearizer and recorder. A molar extinction coefficient of $3.3 \cdot 10^3$ for NADPH was used for calculation of enzyme activity at 20°C. The reaction was carried out in 1 ml of 20 mM imidazole/HCl buffer, pH 7.0, containing 0.5 mM EDTA, 1 mM dithiothreitol, 0.2 mM NADH and 0.8 mM dihydroxyacetone phosphate. All enzyme solutions assayed were desalted by gel filtration (Sephadex G-25), interfering enzyme activities were assayed in the reaction mixtures recommended by Bergmeyer [10]. Protein was determined according to the method of Lowry et al. [11].

The degree of purity was assessed with the inactive enzyme by electrophoresis on 7.5% polyacrylamide gels [12] in the presence of 2% sodium dodecyl sulfate after reduction of the protein with 1% 2-mercaptoethanol. Enzyme activity was detected using 5% polyacrylamide disc gels [13]. Gel tubes were incubated in a solution containing 10 mM sodium α-D,L-glycerolphosphate, pH 7.0, 50 mM hydrazinechloride, 2 mM NAD^+ and 1 mM dithiothreitol and the formation of NADH was followed in a gel scanner (Gilford) at 340 nm.

DEAE-cellulose, DE52, was obtained from Whatman (Springfield Mill, U.K.),

DEAE-Sephadex A-50 from Pharmacia (Upsalla, Sweden), hydroxyapaptite from Bio-Rad (Richmond, CA) and the Diaflo PM-30 membrane from Amicon (Lexington, MA). Streptomycin sulfate, nucleotides and enzymes were products of Boehringer, Mannheim (F.R.G.) and dihydroxyacetone phosphate was obtained as the dimethylketal salt from Sigma, Munich (F.R.G.). All other chemicals were of analytical grade.

Results

Isolation of α-L-glycerolphosphate dehydrogenase

Preliminary experiments established that there was no significant change in the cellular activity of α-L-glycerolphosphate dehydrogenase during growth on glucose. Therefore the enzyme was isolated from cells that had reached the stationary phase after 48 h of aerobic incubation. Isoenzymes were not detected after electrophoresis of extracts from cells of different growth phases on analytical 5% polyacrylamide gels [13]. The cells were washed twice with distilled water, suspended in a solution containing 0.2 mM phenylmethanesulfonylfluoride, 2 mM EDTA and 14 mM mercaptoethanol and disrupted by 2 passes through a French pressure cell. The pH was adjusted to 6.0 and unbroken cells and debris were removed by centrifugation (20 000 · *g*; 30 min). Nucleic acids were precipitated with 0.7% (w/v) streptomycin sulfate. The supernatant solution was buffered with 50 mM triethanolamine/HCl to pH 7.0 and solid ammonium sulfate was added to 40% saturation. After centrifugation (20 000 · *g*; 15 min) the precipitate was discarded. α-L-Glycerolphosphate dehydrogenase was almost quantitatively precipitated by adding ammonium sulfate to the supernatant fraction to reach 55% saturation. The pellet was dissolved in 10 mM triethanolamine/HCl, pH 7.0, with 14 mM mercaptoethanol, 1 mM EDTA and 0.2 mM phenylmethansulfonylfluoride (buffer A), desalted by dialysis against buffer A overnight and then applied to a DE52-cellulose column, which had been previously equilibrated with buffer A. The proteins were eluted with a linear salt gradient of up to 0.3 M ammonium sulfate. Fractions with α-L-glycerolphosphate dehydrogenase activity (eluted at approx. 0.2 M salt) were pooled and dialysed against buffer A. Subsequent chromatography was performed on a DEAE-Sephadex A-50 column, also equilibrated with buffer A. The enzyme emerged at 0.3 M ammonium sulfate in a gradient containing up to 0.5 M salt. After the enzyme had been concentrated with a Diaflo PM-39 membrane in a stirred cell, the protein was dialysed against 10 mM potassium phosphate, pH 6.8, containing 0.5 mM EDTA, 0.2 mM phenylmethansulfonylfluoride and 14 mM mercaptoethanol (buffer B) and bound to a hydroxyapatite column. The enzyme was eluted at 0.1 M salt in a linear gradient containing from 0.01 to 0.3 M potassium phosphate, pH 6.8. Fractions with α-L-glycerolphosphate dehydrogenase activity were pooled and concentrated by diafiltration in a stirred cell (PM-30 membrane). After dialysis against buffer A to remove phosphate the enzyme was stored in 10% glycerol and 1 mM dithiothreitol at 6°C without significant loss of activity over three months.

By this method α-L-glycerolphosphate dehydrogenase was purified 400-fold (Table I). Activities of triose phosphate isomerase and glycerolphosphatase

TABLE I

PURIFICATION OF YEAST α-L-GLYCEROPHOSPHATE DEHYDROGENASE

The enzyme was isolated from 600 g wet wt. *S. carlsbergensis.*

Preparation steps	Total protein (mg)	Total activity (U)	Specific activity (U/mg protein)	Purification (-fold)
Crude extract	47 100	4898	0.104	
Streptomycin sulfate	30 000	3849	0.128	1.2
Ammonium sulfate pellet (40—55%)	7 200	3024	0.42	4
DEAE-cellulose	690	1449	2.1	20.2
A-50	109	1308	12	115
Hydroxyapatite	8	340	42.5	408.7

were reduced to 0.1% and glyceraldehyde-phosphate dehydrogenase to 0.05% compared with glycerolphosphate dehydrogenase. Alcohol dehydrogenase was not detectable. The old hypothesis of Negelein and Brömel [14], that glycerol production in yeast can be attributed to a secondary activity of alcohol dehydrogenase, was thereby clearly disproved. Electrophoresis of the native enzyme on 5% analytical polacrylamide gels reveals one major protein band only, which coincides with α-L-glycerolphosphate dehydrogenase activity. After reduction with mercaptoethanol one major and two minor protein bands of different molecular weight (30 000—50 000) appear in electrophoresis on 7.5% polyacrylamide gels in the presence of 2% sodium dodecyl sulfate, which may indicate that the enzyme is not yet homogeneous.

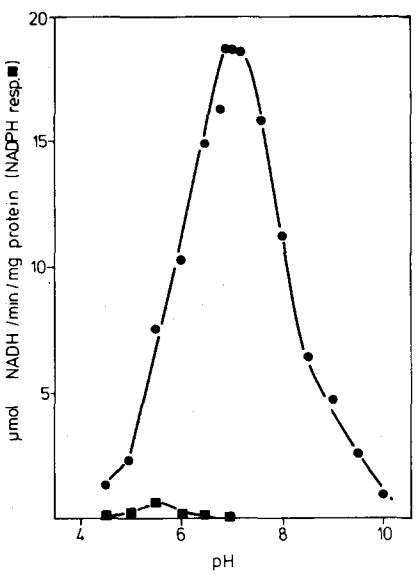

Fig. 1. pH-optimum curve of α-L-glycerolphosphate dehydrogenase with 0.2 mM coenzyme and 0.8 mM dihydroxyacetone phosphate. One curve (●———●) indicates the activity of the enzyme with NADH, the other curve (■———■) the activity with NADPH.

Substrate specificity

α-L-glycerolphosphate dehydrogenase from *S. carlsbergensis* is specific for dihydroxyacetone phosphate at pH 7.0. Glyceraldehyde and dihydroxyacetone are not converted, neither with NADH nor with NADPH. The limited reduction of glyceraldehyde phosphate observed (0.1% of the activity with dihydroxyacetone phosphate) is explained by contamination with traces of triosephosphate isomerase. With the method of Cleland [15] a K_m of 0.203 ± 0.027 mM was determined under standard asay conditions.

At pH 7.0, the enzyme is strictly specific for NADH (Fig. 1). A K_m of 0.024 ± 0.004 was determined with 0.8 mM dihydroxyacetone phosphate. There is a remarkable pH dependency of the coenzyme specificity, which has been already described for other dehydrogenases [16]. As is evident from Fig. 1, the enzyme reduces NADPH at a very low rate in the pH range between 5.0 and 6.0. We observed the same effect with α-L-glycerolphosphate dehydrogenase from rabbit muscle with a pH optimum for NADH between 7.5 and 9.0 and for NADPH between 5.0 and 7.0.

Influence of ions

α-L-Glycerolphosphate dehydrogenase is inhibited by a number of ions (Table II). Contrary to the suggestion of Gancedo [3] there appears to be no general ion inhibition. Neither the concentration nor the identity of the cation is

TABLE II

EFFECTS OF IONS ON YEAST α-L-GLYCEROPHOSPHATE DEHYDROGENASE

The concentrations of NADH (0.2 mM) and dihydroxyacetone phosphate (0.8 mM) were held constant.

Salt	Concentration (mM)	Residual activity (%)	Mean hydration number of the cation	Ionic strength
Chloride salts				
LiCl	30	50	4	0.03
NaCl	30	33	3	0.03
KCl	30	40	2	0.03
CsCl	30	33		0.03
$MgCl_2$	30	15		0.09
$MgCl_2$	15	40		0.045
$CaCl_2$	30	15		0.09
$CaCl_2$	15	40		0.045
NH_4Cl	30	40		0.03
Sodium salts				
NaF	30	100	3	0.03
NaCl	30	33	2	0.03
NaBr	30	20		0.03
NaI	30	10	0.7	0.03
Na_2SO_4	30	19		0.09
NaSCN	30	18		0.03
$NaNO_3$	30	3		0.03
Sodium citrate	30	20		0.36
Sodium phosphate	30	0		0.067
Sodium pyrophosphate	30	0		
Sodium pyrophosphate	4	50		
Sodium arsenate	30	0		
Sodium arsenate	1	60		

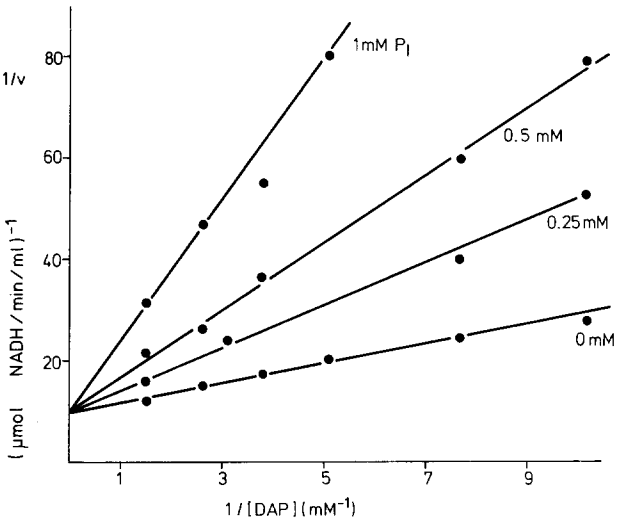

Fig. 2. Lineweaver-Burk plot of the reciprocal activity of α-L-glycerolphosphate dehydrogenase with varied dihydroxyacetone phosphate concentrations, showing the effect of varied phosphate concentrations. NADH concentration was held constant at 0.2 mM.

correlated with the strength of inhibition; the identity of the anion seems to be the decisive factor. Inhibition decreases with increasing hydration ($F^- > Cl^- > Br^- > I^-$). Phosphate and similarily arsenate and pyrophosphate are the strongest inhibitors, with phosphate inhibiting the enzyme competitively towards dihydroxyacetone phosphate as shown in Fig. 2. From the corre-

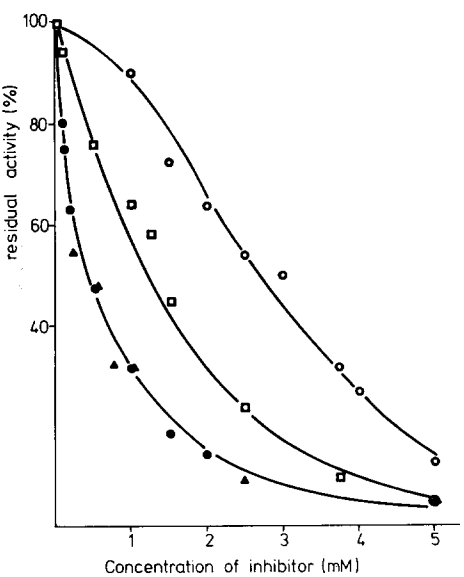

Fig. 3. Effect of phosphate (●), ATP (▲), $MgATP^{2-}$ (□), and $MgADP^-$ (○) on the activity of α-L-glycerolphosphate dehydrogenase. NADH (0.2 mM) and dihydroxyacetone phosphate (0.8 mM) concentrations were held constant.

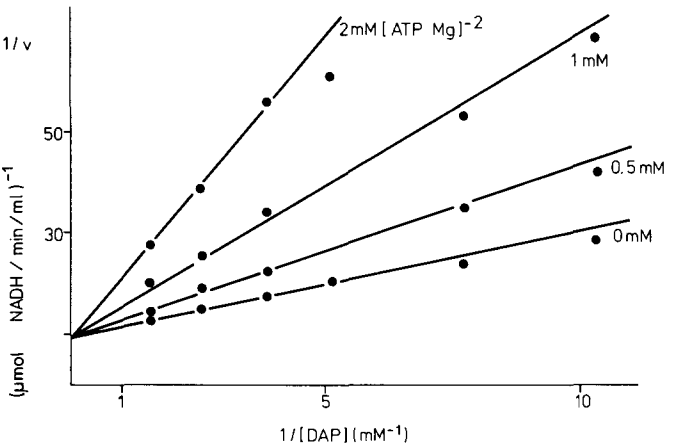

Fig. 4. Lineweaver-Burk plot of the reciprocal activity of α-L-glycerolphosphate dehydrogenase with varied dihydroxyacetone phosphate and constant NADH (0.2 mM) concentration, showing the effect of varied $MgATP^{2-}$ concentrations on the enzyme activity.

sponding Dixon plot a K_i value of 0.1 mM was calculated with NADH held constant at 0.2 mM. Inhibition by P_i is noncompetitive towards NADH with 0.8 mM dihydroxyacetone phosphate. The chloride anion also competes with dihydroxyacetone phosphate for the active site, but with a much higher dissociation constant (K_i = 2.5 mM).

Inhibition by nucleotides

The enzyme is inhibited by ADP and ATP (Fig. 3). In intact cells, ATP and ADP are largely present as the $MgATP^{2-}$ and $MgADP^{-}$ complexes because of the high affinity of the pyrophosphate groups toward divalent cations. These complexes inhibit α-L-glycerolphosphate dehydrogenase less than do the uncomplexed nucleotides. We suppose that α-L-glycerolphosphate dehydrogenase is inhibited by the phosphate groups of ADP and ATP. This assumption is supported by the observation that inhibition by phosphate and $MgATP^{2-}$ are of the same type (Fig. 4). For $MgATP^{2-}$ a K_i of 0.73 mM was determined with 0.2 mM NADH.

Discussion

Similar to α-L-glycerolphosphate dehydrogenase from other sources, the yeast enzyme is specific for NADH. There exists, however, a small activity with NADPH limited to the pH range 5.0—6.0. Since our preparation is essentially free of contaminating enzymes, we consider this activity to be due to the action of α-L-glycerolphosphate dehydrogenase. Even in a crude preparation, from which salts and metabolites have been removed by gel filtration, the maximal activity of α-L-glycerolphosphate dehydrogenase is 4.6 µmol/g wet wt./min with NADH and 0.13 µmol/g/min for NADPH. As glycerol is produced with a rate of 2.5 µmol/g/min in *Saccharomyces* cells [5] it is evident that NADH must be the coenzyme responsible for glycerol production in vivo and

not NADPH. The possibility that the pentose phosphate pathway serves as the major H donor for glycerol synthesis, suggested by Kreuzberg [6], may therefore be disregarded.

In spite of this observation the turnover rate in the pentose phosphate shunt may nevertheless be loosely correlated with glycerol production by means of control of the cellular redox potential mediated by the α-L-glycerolphosphate dehydrogenase reaction. Yeast cells can grow anaerobically using energy from glycolysis exclusively. For synthesis of lipids, amino acids, nucleic acids and other cell constituents metabolites are withdrawn from the glycolytic and other pathways. Therefore acetaldehyde, which is needed for the reoxidation of NADH, will not be formed in an amount equivalent to NADH, since acetyl-CoA is diverted to serve biosynthetic needs. The result will be a shortage of NAD^+, limiting the reaction catalyzed by glyceraldehyde-phosphate dehydrogenase. This shortage in NAD^+ can be corrected by glycerol production at a rate dependent upon the rate of biosynthesis and the flux in the pentose phosphate pathway, which yields NADPH for anabolic reactions.

There are few available data on the concentrations of anions in yeast. Lichko et al. [17] estimate phosphate to be approx. 1 mM in the cytoplasm. We can expect that the dehydrogenase will be drastically inhibited in this concentration range. Since anion inhibition is competitive with dihydroxyacetone phosphate, the actual rate of reduction will depend on the concentration of the triose phosphate. In the presence of high NADH (and low NAD^+) concentrations glyceraldehyde-phosphate dehydrogenase is inhibited, with a consequent increase of dihydroxyacetone phosphate. Its accumulation is further enhanced if 6-phosphofructokinase is activated by low ATP and high ADP and AMP concentrations. With higher dihydroxyacetone phosphate concentrations α-L-glycerolphosphate dehydrogenase becomes more and more active, permitting the reoxidation of NADH. In this way the redox potential of $NADH/NAD^+$ may be regulated at α-L-glycerolphosphate dehydrogenase. Under conditions of high energy charge the glycolytic flux is limited at 6-phosphofructokinase. With falling dihydroxyacetone phosphate concentrations α-L-glycerolphosphate dehydrogenase again becomes less active. Perhaps direct inhibition by ATP will enhance the effect of phosphate inhibition. The ADP concentration in the cytoplasm of actively fermenting yeast is too low to permit an inhibition of the enzyme [18].

In muscle and in yeast α-L-glycerolphosphate is consumed aerobically by a mitochondrial oxidase. In yeast, which is able to grow under anaerobic conditions, the accumulation of α-L-glycerolphosphate and glycerol is prevented by the sensitivity of α-L-glycerolphosphate dehydrogenase to anions and nucleotides. Its activity is in balance with the $NADH/NAD^+$ system in so far as it allows the reoxidation of NADH but keeps the loss of dihydroxyacetone phosphate to a minimum.

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