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NADP-Glutamate Dehydrogenase Activity Is Increased under Hyperosmotic Conditions in the Halotolerant Yeast *Debaryomyces hansenii*

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Abstract. Glutamate plays an important role in osmoprotection in various bacteria. In these cases, increased intracellular glutamate pools are not attributable to the NADP-dependent glutamate dehydrogenase (NADP-GDH) or the glutamate synthase, which do not increase their activities under hyperosmotic conditions, but rather to changes in other enzymes involved in glutamate metabolism. We performed a study which indicates that, as opposed to what happens in bacteria, the activity of NADP-GDH is fivefold higher when the halotolerant yeast *Debaryomyces hansenii* is grown in the presence of 1 M NaCl, compared with growth in media with no added salt. Since purified NADP-GDH activity in vitro was not enhanced by the presence of salt and was more sensitive to ionic strength than the two isoenzymes from *S. cerevisiae*, increased enzyme synthesis is the most plausible mechanism to explain our results. We discuss the possibility that increased NADP-GDH activity in *D. hansenii* plays a role in counteracting the inhibitory effect of high ionic strength on the activity of this enzyme.

Most free-living microorganisms possess amino acid biosynthetic pathways that allow the cell to use ammonium as sole nitrogen source. Ammonium utilization occurs exclusively via its incorporation into glutamate and glutamine [14]. This process can be achieved by two metabolic routes, one of them constituted by the concerted action of glutamine synthetase and the *GLT1* encoded glutamate synthase (GOGAT) [18]. The other pathway is mediated by the NADP-dependent glutamate dehydrogenase (NADP-GDH), EC 1.4.1.4, the enzyme that catalyzes the reductive amination of 2-oxoglutarate to form glutamate [12]. Most microorganisms possess a single gene encoding NADP-GDH. However, in *Saccharomyces cerevisiae*, two genes (*GDH1* and *GDH3*) have been described whose products constitute NADP-GDH isoenzymes [2, 9]. When cells are exposed to high osmolarity, they respond by accumulating and/or synthesizing a compatible solute inside the cell in order to counterbalance higher external osmotic pressure [7, 19].

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It has been shown that in various bacteria glutamate accumulates to act as an osmolyte. When *Salmonella typhimurium* is grown on 500 mM NaCl, the glutamate pool increases threefold as compared with that found without salt [6, 8]. *Rhizobium meliloti* is also able to accumulate glutamate during osmotic stress [5]. However, glutamate accumulation has not been shown to be the result of increased NADP-GDH nor GOGAT activities, but it is rather due to the action of other enzymes involved in glutamate metabolism [6].

Debaryomyces hansenii is a spoilage yeast found as a contaminant of brine food; it displays the capacity to grow in media containing a wide range of salt concentrations, including sea water, from which it has been isolated [15]. Exposition to a high environmental osmolarity leads to dehydration of cells and decreases viability. To overcome this, cells have developed mechanisms to adapt to critical osmotic changes in their environments [3]. Osmoregulation is a complex cellular response, and many efforts have been made to understand the molecular mechanism of this phenomenon. In *D. hansenii* it

has been shown that glycerol and arabitinol are the main osmolytes involved in salt resistance and that amino acids play no role in this process [1, 19].

We have studied the effect of high salt concentration on the activity and kinetic parameters of the NADP-GDH, in order to analyze the effect of high ionic strength on this enzyme during growth under hyperosmotic conditions. We considered the possible existence of mechanisms allowing enzyme activity when the intracellular ion concentration is greatly increased. Our results show that NADP-GDH activity is increased in *D. hansenii* when the yeast is grown on hyperosmotic conditions, and that this activity is inhibited by ionic strength and is more sensitive to this effect than the two isoenzymes from *S. cerevisiae*.

Materials and Methods

Strains and growth conditions. *D. hansenii* and *S. cerevisiae* wild-type strains Y7426 and S288C, respectively, were used throughout this work. Cells were routinely grown on rich medium containing 2% glucose, 1% yeast extract (Difco), and 2% peptone (Difco) (YPD). Several formulas for minimal medium were tested: i) MM1, containing salts, trace elements, and vitamins following the formula of yeast nitrogen base (Difco) with 2% glucose; ii) MM2, prepared following the formula described by Norkrans [16]; 0.5% $(NH_4)_2SO_4$ (wt/vol) or 0.1% (wt/vol) of asparagine or glutamate was used as nitrogen source as stated in the text. Cells were routinely grown at 30°C with shaking (180 rpm). For solid media 2% (wt/vol) agar was added. Growth was monitored by measuring optical density at 600 nm. In all cases, growth in media with added NaCl at the molar concentrations stated in the text was compared with growth in media without salt.

Preparation of cell-free extracts and NADP-GDH determinations. Cells were harvested by centrifugation from mid-exponentially grown cultures, washed in deionized water, and resuspended in 1.5 mL of 0.1 M potassium phosphate pH 7.4, 1 mM EDTA [10, 11]. Cells were ground by vortexing with glass beads, and the suspension was spun in a microcentrifuge tube for 10 min at 12,000 rpm at 4°C. This extract was kept on ice and used to assay enzyme activity. NADP-GDH activity was assayed as described by Doherthy [10]. Protein was measured following the method described by Lowry et al. [13], with bovine serum albumin as a standard. NADP-GDH activities from cells grown in media with added NaCl were compared with the activities observed in cells grown in media without salt.

NADP-GDH purification. NADP-GDH was purified from a 30-liter culture of *D. hansenii* grown on MM2 with asparagine as nitrogen source in the presence of 1.0 M NaCl in the culture medium. Cells were harvested from mid-exponential growth phase cultures. NADP-GDH purification was carried out following the method described by DeLuna et al. [9]. In order to elute the extract from the Reactive Red agarose column, a buffer containing 0.3 mM NADPH was used instead of that previously described [9], which contains 0.1 mM NADPH. Fractions with activity were pooled and dialyzed against 20 mM Tris-HCl pH 7.5, 1.0 mM EDTA, and concentrated by ultrafiltration to ~1 mg/mL with Amicon YM30 membrane and stored at −70°C until used.

Enzyme kinetics. NADP-GDH activity was assayed at different concentrations of 2-oxoglutarate, NADPH, or ammonium chloride and at saturating concentrations of the remaining substrates (7.5 mM 2-oxoglutarate, 125 mM ammonium chloride, and 0.15 μM NADPH). The progress of the reaction was always kept below 5% conversion of the initial substrate. Measurements were made at 25°C in 100 mM Tris at pH 8.0. For experiments in which pH was varied, citric acid was used to adjust the reaction buffer. The K_M and V_{max} were obtained from an Eadie-Hofstee plot.

Table 1. Doubling time for *D. hansenii* and *S. cerevisiae* cultivated under increasing concentrations of NaCl

[NaCl] (M)	Doubling time (hours)	
	D. hansenii	*S. cerevisiae*
0.0	4.0	2.0
0.5	3.8	3.0
1.0	4.0	5.0
2.0	8.5	15.0
2.5	22.0	∞

Cells were grown on MM2 supplemented with asparagine.
∞, no detectable growth.

Effect of ionic strength on NADP-GDH activity in vitro. Purified enzyme and cell extracts prepared from yeasts grown in the absence and presence of salt (1.0 M), and purified enzyme were treated with increasing concentrations of NaCl and KCl.

Glutamate pool determination. Intracellular glutamate content was determined following the method described by Bergmeyer [4].

Electrophoresis and immunoblotting. SDS polyacrylamide gel electrophoresis was performed with 10% slab gels. Proteins on polyacrylamide gels were visualized with Coomassie Blue. Immunoblot analysis of SDS-electrophoresed from *S. cerevisiae* crude extracts and pure NADP-GDH from *D. hansenii* was carried out as described by Towbin et al. [20], with *S. cerevisiae* NADP-GDH antiserum [9].

Results

Growth of *D. hansenii* on high NaCl concentrations and asparagine as nitrogen source. Growth of a prototrophic strain of *D. hansenii* under different NaCl concentrations was assayed on MM2 plus asparagine and glucose and compared with growth in media with no added NaCl (Table 1). No substantial difference in growth was observed in the absence of salt or with 0.5 or 1.0 M NaCl. However, when 2.0 or 2.5 M NaCl was used, the generation time was increased in a concentration-dependent manner. On the other hand, growth of *S. cerevisiae* was affected even in the presence of 0.5 M NaCl and was completely inhibited at 2.5 M NaCl.

To determine which nitrogen source allowed optimal growth, ammonium sulfate, the 20 amino acids, and the four nitrogen bases were used as sole nitrogen sources in MM1 or MM2. Optimal growth was found on MM2 in the presence of either asparagine or glutamate (data not shown), confirming previous observations showing that, as opposed to what happens in *S. cerevisiae*, asparagine or glutamate is a better nitrogen source than ammonium for *D. hansenii* [16].

Table 2. Effect of the presence of NaCl on NADP-GDH activity

Culture medium[a]	NADP-GDH (Sp. Act.)[b] without NaCl	with NaCl
YPD	N.D.[c]	0.034 ± 0.005
MM2 + amm	0.122 ± 0.027	0.207 ± 0.073
MM2 + Glu	0.086 ± 0.003	0.217 ± 0.001
MM2 + Asn	0.110 ± 0.013	0.510 ± 0.098

[a] Cells were grown on MM2 supplemented with ammonium sulfate, glutamate, or asparagine.
[b] Values are presented as means from three independent experiments ± S.D.
[c] N.D., not detectable.

NADP-GDH activity and Western analysis under different growth conditions. NADP-GDH enzymatic activity was assayed in extracts from *D. hansenii* cultured in different media with and without addition of NaCl (Table 2). In extracts prepared from yeast grown on YPD, enzymatic activity was not detectable. However, in the presence of 1 M NaCl, activity was increased, suggesting the production of a higher amount of enzyme. Accordingly, highest activities were determined in extracts from MM2 with either ammonium, asparagine, or glutamate (Table 2). A fivefold increment in NADP-GDH activity was attained on asparagine plus 1.0 M NaCl as compared with that found without salt. These results indicate that, in the presence of salt, a higher synthesis and/or activity of NADP-GDH is attained.

Although increased NADP-GDH activity in the presence of salt could result in a higher glutamate pool, it was found that the intracellular glutamate concentrations were 9 nmol mg^{-1} of wet weight in the presence or absence of salt. Thus, it can be concluded that the increased NADP-GDH activity does not result in increased glutamate pools.

Western analysis of cell-free extracts of *D. hansenii* with *S. cerevisiae* from NADP-GDH antiserum showed a single band, suggesting the existence of a single NADP-GDH enzyme in this yeast (Fig. 1). Furthermore, a higher amount of antigen was detected in extracts obtained from cells grown in the presence of salt, compared with the immunochemical signal detected in extracts from cultures without NaCl.

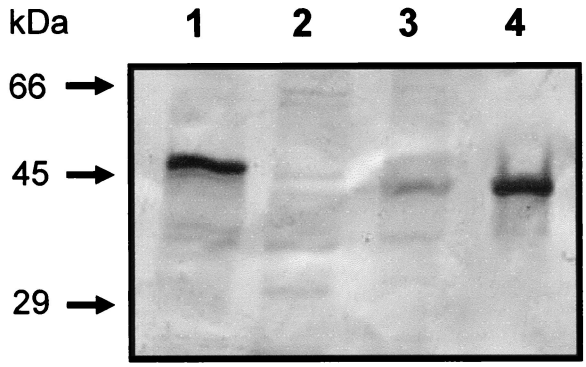

Fig. 1. Immunoblot analysis of NADP-GDH from *S. cerevisiae* and *D. hansenii*. *S. cerevisiae* was grown on MM1 with ammonium, and *D. hansenii* was grown on MM2 with asparagine. Lane 1, crude extract from *S. cerevisiae* cells grown on MM1 without NaCl; Lane 2, crude extract from *D. hansenii* grown on MM2 supplemented without NaCl; Lane 3, crude extract from *D. hansenii* grown on MM2 with 1.0 M NaCl; Lane 4, purified NADP-GDH from *D. hansenii* grown on MM2 with 1.0 M NaCl.

Enzyme kinetics and salt sensitivity of NADP-GDH from *D. hansenii*. In order to study the sensitivity of NADP-GDH from *D. hansenii* to ionic strength, the enzyme was 43-fold purified following the previously described procedure [9]. Kinetic parameters of NADP-GDH from yeast grown in the presence or absence of salt were determined in the purified preparation and in whole-cell extracts. Both preparations showed similar K_M values for all substrates, suggesting that the same enzyme is present in cultures prepared with or without salt (Table 3). The effect of pH was assayed; as expected, a bell-shape curve was obtained for NADP-GDH activity determined in crude extracts from cultures of the yeast grown either with or without 1.0 M of NaCl. The optimal pH was found to be 8.0 for both extracts (data not shown).

The effect of ionic strength on the activity of purified NADP-GDH was tested with NaCl and KCl (Fig. 2).

Table 3. Kinetic parameters of NADP-GDH from *D. hansenii* obtained from different growth conditions

Condition[a]	2-oxoglutarate K_M (mM)	2-oxoglutarate V_{max} (μmol min^{-1} mg^{-1})	Ammonium K_M (mM)	Ammonium V_{max} (μmol min^{-1} mg^{-1})	NADPH K_M (μM)	NADPH V_{max} (μmol min^{-1} mg^{-1})
−NaCl, extract	1.4	0.24	17.0	0.26	39.0	0.19
+NaCl, extract	1.7	0.66	12.0	0.56	38.4	0.36
+NaCl, purified	2.2	21.0	16.0	14.0	21.0	15.5

[a] Cells were grown on MM2 supplemented with asparagine, with or without 1.0 M NaCl.

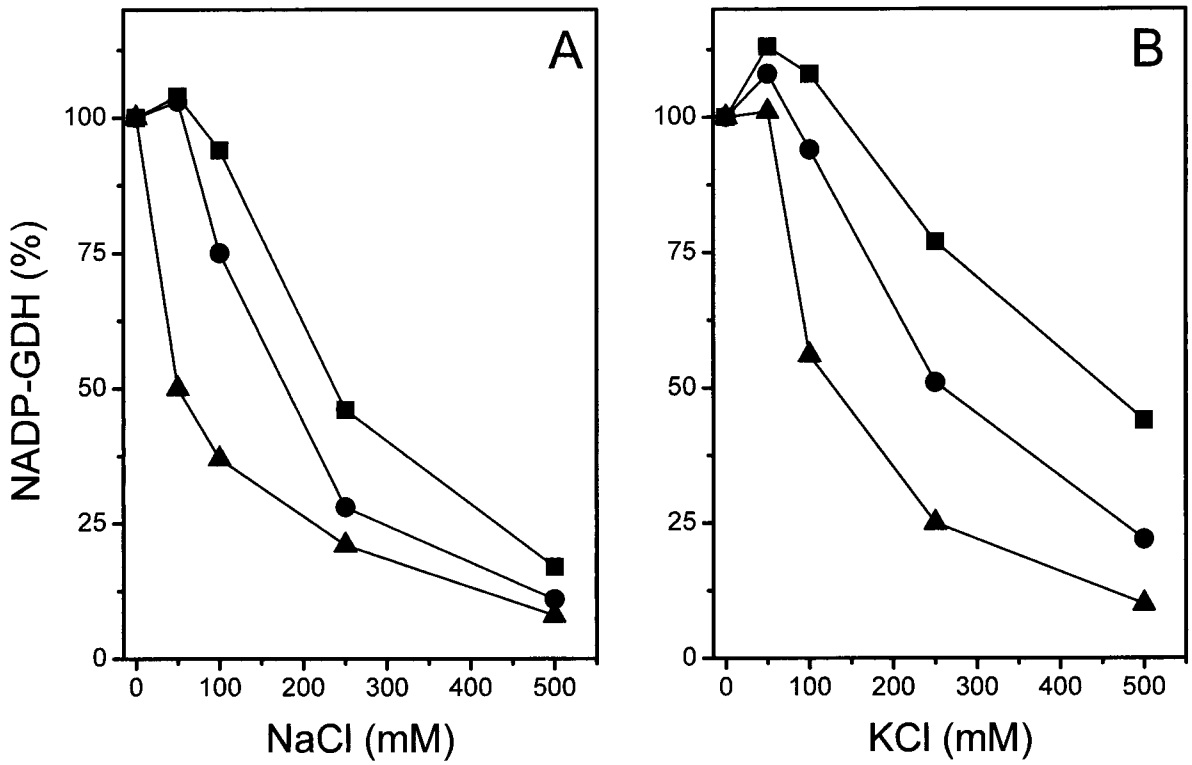

Fig. 2. Effect of ionic strength on purified preparations of NADP-GDH from *S. cerevisiae* and *D. hansenii*. Plots show the sensitivity of *S. cerevisiae* and *D. hansenii* enzymes to increasing (A) NaCl or (B) KCl concentrations. ■, Gdh1p from *S. cerevisiae;* ●, Gdh3p from *S. cerevisiae;* ▲, Gdhp from *D. hansenii*.

At low ionic strength, a clear difference in sensitivity was observed between the enzyme from *D. hansenii* and those of *S. cerevisiae*. At 50 mM NaCl, both *S. cerevisiae* isozymes retained full activity, while the enzyme from *D. hansenii* lost 50% of its initial activity. A strong difference in salt sensitivity was also observed at 100 mM NaCl. Treatment with KCl showed similar results, although the enzymes were more resistant to this salt. Interestingly, when ionic strength was increased with NaCl (500 mM), the three enzymes were similarly inhibited (90%). However, 500 mM KCl equivalently inhibited *D. hansenii* NADP-GDH and *S. cerevisiae* Gdh3p (80–90%), while Gdh1p retained 50% activity (Fig. 2). Taken together, these results indicate that NADP-GDH from *D. hansenii* is inhibited by ionic strength and is more sensitive to this effect than the two isoenzymes from *S. cerevisiae*.

Discussion

The results presented in this paper confirm that glutamate is not accumulated when *D. hansenii* is grown in the presence of NaCl. However, NADP-GDH activity increased fivefold under hyperosmotic conditions. Additionally, our results show that the NADP-GDH enzyme from this yeast was inhibited by ionic strength. Taken together, these results suggest that a higher NADP-GDH activity could be needed in order to counteract the inactivation resulting from the accumulated ions. This would build up a higher enzymatic pool that could buffer the inhibitory effect of ionic concentration, given that intracellular Na^+ increases up to 600 mM in *D. hansenii* [17].

The increase in NADP-GDH activity when this yeast is grown in the presence of NaCl could be due to: i) an increase in enzyme synthesis, ii) activation of the enzyme by the presence of salt, or iii) a differential activation of a GDH-coding gene that would be expressed only in the presence of salt. The latter possibility occurs in *S. cerevisiae*, which expresses *GDH3* when the yeast is grown on ethanol as carbon source [9]. Our results indicate that increased enzyme synthesis is the most plausible mechanism to explain the obtained results, since enzyme activity was not enhanced in the presence of salt, and Western analysis revealed the presence of a higher amount of protein under hyperosmotic conditions. Furthermore, we did not detect the presence of two NADP-GDH isoenzymes in this yeast. Whether increased enzyme synthesis is due to increased transcrip-

tion of the gene coding for this enzyme remains to be analyzed.

An ample study of the effect of ionic strength on the activity of the enzymes involved in intermediary metabolism in *D. hansenii* would shed light on the possibility that increasing enzymatic activities constitute a general mechanism involved in osmoadaptation.

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