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Osmoregulation in *Saccharomyces cerevisiae*

Studies on the osmotic induction of glycerol production and glycerol 3-phosphate dehydrogenase (NAD^+)

Lars André, Anna Hemming and Lennart Adler

Department of General and Marine Microbiology, University of Göteborg, Carl Skottsbergsg. 22, S-413 19 Göteborg, Sweden

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Production of glycerol and a key enzyme in glycerol production, glycerol 3-phosphate dehydrogenase (NAD^+) (GPD), was studied in *Saccharomyces cerevisiae* cultured in basal media or media of high salinity, with glucose, raffinose or ethanol as the sole carbon source. At high salinity, glycerol production was stimulated with all carbon sources and glycerol was accumulated to high intracellular concentration in cells grown on glucose and raffinose. Cells grown on ethanol accumulated glycerol to a lower level but showed an increased content of trehalose at high salinity. However, the trehalose concentration corresponded only to about 20% of the glycerol level, and did not compensate for the shortfall in intracellular osmolyte content. Immunoblot analysis demonstrated an increased production of GPD at high salinity. This increase was osmotically mediated but was lower when glycerol was substituted for NaCl or sorbitol as the stress-solute. The enzyme also appeared to be subject to glucose repression; the specific activity of GPD was significantly lower in cells grown on glucose, than on raffinose or ethanol.

Saccharomyces cerevisiae; Glycerol; Osmoregulation; Glycerol 3-phosphate dehydrogenase; Trehalose

1. INTRODUCTION

The mechanisms by which microorganisms adapt to osmotic stress have received considerable interest (for reviews, see [1,2]. A general response to exposure to lowered water potential is a rapid intracellular accumulation of compensatory osmolytes [3]. Although any solute can be used to lower the water potential, only a restricted group of so-called compatible solutes are adopted for the intracellular osmotic adjustments. A substantial body of evidence indicates that polyols, in particular glycerol, have a role as compatible solutes in fungi [1,4–9]. In *Saccharomyces cerevisiae*, glycerol was recognized as a compatible solute based on the observation that increased production and intracellular accumulation correlated with decreased water potential of the medium [1].

Although it is well established that *S. cerevisiae* accumulates glycerol in response to osmotic stress, it is difficult to ascertain the role of glycerol production in osmoregulation when glucose is used as the carbon source. This is due to the fact that glycerol formation plays an important role in the oxidation of NADH during growth in glucose media [10,11] due to glucose repression of the respiratory capacity [12–15]. Glycerol is synthesized by reduction of dihydroxyacetone phosphate to glycerol 3-phosphate by glycerol 3-phosphate dehydrogenase (NAD^+)(EC 1.1.1.8; GPD), followed by dephosphorylation of glycerol 3-phosphate to glycerol [16]. Hence, the glycerol 3-phosphate dehydrogenase functions at the metabolic branch point between the glycolitic sequence and the glycerol synthesizing pathway. It was observed by Edgley and Brown [17], that the activity of GPD of *S. cerevisiae* increased in cells grown in media of diminished water potential. However, the molecular level at which this activation took place remained unsolved. Using immunoblot analysis, we have examined the production of GPD in response to osmotic stress under various growth conditions. We also report on the osmotically induced production and accumulation of glycerol under conditions in which glucose repression does not occur, such as with raffinose or ethanol as the sole carbon source.

Correspondence address: L. Adler, Department of General and Marine Microbiology, University of Göteborg, Carl Skottsbergsg. 22, S-413 19 Göteborg, Sweden

2. EXPERIMENTAL

2.1. *Yeast strain and culture conditions*

S. cerevisiae strain S288C (α SUC2 *mal mel gal*2 CUP1) was routinely grown in 2.8 liter Fernbach flasks, containing 500 ml Yeast Nitrogen Base (Difco), supplemented with 2% (w/v) glucose, raffinose (Ridel de Haen AG, The Netherlands) or ethanol. The flasks were incubated at 30°C on a rotary shaker and cultures were grown until the cell density reached $A_{610} = 1$. The harvested cells were washed and transferred to basal medium or to medium supplemented with NaCl (0.34, 0.68 or 1.02 M NaCl). Where indicated NaCl (0.68 M) was substituted by isotonic concentrations of glycerol or sorbitol (1.11 M). Although, these additions decreased the water potential to

similar extents (by 3.05 MPa at 20°C [18]), effects due to carbon source have to be taken into account. Glucose and raffinose would maximally (at 2%, w/v) lower the water potential by 0.27 MPa and 0.08 MPa, respectively, whereas ethanol would cause a decrease of 1.05 MPa. Since the effects by the two sugar substrates were relatively small and since ethanol, due to rapid equilibration through the yeast plasma membrane [19], is likely to not increase the osmotic gradient over the membrane, the effects due to carbon source will not be further considered.

The pH of all media was adjusted to 6.0. Dry weights were determined with cells washed twice in distiled water and dried at 95°C for 48 h.

2.2. *Extraction and analysis of glycerol and trehalose*

Glycerol was analyzed with a commercial glycerol assay kit (Boehringer Mannheim, Germany) and the intracellular content of glycerol was determined as the difference between total and extracellular glycerol of centrifuged heat-treated samples, as previously described [5].

Trehalose was extracted from 1.5 mg dry wt. yeast which was sampled on a Millipore HAWP filter and washed with 20 ml of isotonic NaCl solution. Cells were extracted for 2 h at 75°C with 4 ml of 80% v/v of ethanol containing phenyl β-D-glucoside as an internal standard. Dried samples were each resuspended in 100 μl 80% (v/v) ethanol and applied to a C18 cartridge (Bond Elut; Analytrichem International, USA) which was eluted with 500 μl 80% (v/v) ethanol. The eluate was lyophylized and stored frozen until analyzed. The lyophylized sample was silylated and analyzed on a HP5890 (Hewlett-Packard, USA) gas chromatograph equipped with a fused silica capillary column, DB 1701 (30 m by 0.25 mm inner diameter; J and W Scientific, USA).

2.3. *Preparation of cell-free extracts and measurements of enzyme activity*

A cell pellet from a 240 ml culture was suspended in 1 ml TrED buffer) (10 mM triethanolamine, 1 mM EDTA, 1 mM dithiothreitol, pH 7.5) containing 200 μM phenylmethylsulfonylfluoride, 1 μM pepstatin and 1 μM leupeptin. 1 g of glass beads (0.4 mm diameter) was added and the sample was homogenized for 5 min, at 2°C in a Vibrogen Zellmühle disintegrator (Bühler, Germany). The homogenate was centrifuged at $18000 \times g$ for 15 min at 4°C and the supernatant was desalted by passage though a Sephadex G-25 column (Pharmacia PD-10, Pharmacia Fine Chemicals, Sweden). Desalted samples were used immediately for enzymatic assays or treated with lysis buffer for gel electrophoresis [20].

GPD assays and activity calculations were performed as previously described [6] except that assays were performed in TrED-buffer which yielded more stable but somewhat lower specific activities than the previously used buffer system.

Protein concentrations were determined by a Coomassie dye binding technique [21].

2.4. *Preparation of antiserum and purification of antibodies*

Purified GPD from baker's yeast [22] was kindly supplied by Dr. J.R. Merkel and rabbit antiserum was prepared by standard techniques. Immunoinactivation experiments confirmed that the antiserum, as opposed to pre-immunization serum, precipitated the GPD activity of cell-free extracts. Antibodies were further purified with an immunoadsorbent prepared by coupling of 1.5 mg of purified GPD to CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals, Sweden) as recommended by the manufacturer. A 0.5×1.5 cm column of the resulting adsorbent was washed at a flow rate of 3 ml/h with 1.0 M propionic acid followed by 10 mM Na/K-phosphate, 138 mM NaCl and 2 mM KCl, pH 7.2, until the eluate reached the pH of the buffer. Thereafter, 15 ml of serum was pumped through the column. The column was washed with 12 vols. of 2.0 M KCl and eluted by desorption with 1.0 M propionic acid. Fractions containing protein were pooled and frozen after being dialysed against several changes of 10 mM Tris-HCl, 0.15 M NaCl, pH 7.5.

2.5. *SDS-PAGE*

Cell homogenates were mixed with lysis buffer [20] and samples were subjected to discontinuous SDS-PAGE in a 3% stacking and a 7.5-20% (w/v) linear gradient gel containing 0.125% (w/v) SDS in the Laemmli [20] buffer system. Electrophoresis was carried out at 15 mA for 15 h.

2.6. *Immunoblot and antigen detection*

The electrophoresed proteins were transferred (Trans-Blot cell, Bio-Rad, USA) onto nitrocellulose paper (Schleicher and Schuell, USA) in a 25 mM Tris, 192 mM glycine buffer containing 20% (v/v) methanol, pH 8.3 and treated for 1 h in 1% bovine serum albumin in TTBS (20 mM Tris-HCl, 0.05% Tween-20, 500 mM NaCl, pH 7.5). The filters were incubated for 1 h at room temperature with purified antibodies (0.3 mg protein/ml) diluted 1:100 in TTBS. The immune complexes were visualized using goat anti-rabbit alkaline phosphatase conjugated antibodies (Bio-Rad, USA), according to the manufacturers instructions. For determination of molecular weight, the mobility of the immunologically detected proteins was compared to that of molecular weight standards (MW 200 kit, Sigma, USA) that were transferred to nitrocellulose and visualized by staining with Coomassie blue. Immunoblot scanning was performed with an LKB 222-020 Ultroscan XL (Pharmacia, Sweden), using the internal digital integrater system.

3. RESULTS

3.1. *Growth characteristics*

In Fig. 1 the growth curves for *S. cerevisiae* 288C grown in glucose, raffinose or ethanol medium containing 0, 0.34 and 0.68 M NaCl, are depicted. In all cases,

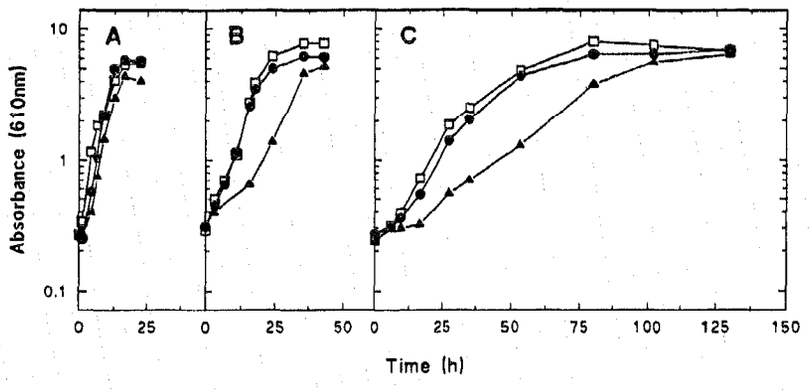

Fig. 1. Growth curves for *S. cerevisiae* cultured in (A) glucose-, (B) raffinose-, or (C) ethanol medium containing 0 M (□), 0.34 M (●), or 0.68 M NaCl (▲).

Table I

Total and intracellular glycerol produced by *S. cerevisiae*, grown in media containing different carbon sources at two concentrations of NaCl

NaCl added (conc.)	Total and intracellular glycerol (μmol/mg dry wt.)					
	Glucose		Raffinose		Ethanol	
	Total	Intra	Total	Intra	Total	Intra
None	5.81 ± 0.4	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
0.68 M	11.6 ± 0.7	1.77 ± 0.3	5.41 ± 1.2	1.22 ± 0.14	1.51 ± 0.16	0.49 ± 0.04

Amounts given represent the mean ± SD of duplicate samples from two or three independent cultures.

the sampling points ($A_{610} = 1$) for analysis of glycerol, trehalose, and specific activity of GPD were within the exponential growth phase. It is also evident that the highest salt concentration had a pronounced effect on the growth kinetics of the ethanol culture, although the final cell density was not appreciably affected by high salt concentration.

3.2. *Glycerol levels*

Cells grown in basal medium on raffinose or ethanol produced less than 0.1 μmol glycerol per mg dry wt. (Table I), whereas cells cultured on glucose produced 5.8 μmol/mg dry wt, essentially all of which being released to the external medium. At increased salinity (0.68 M NaCl) glycerol was produced by all cultures, irrespective of carbon source. Under these conditions the total glycerol production by the cells cultured on raffinose and ethanol was about 50% and 15%, respectively, of that produced by the glucose grown cells. The corresponding intracellular glycerol levels were approx. 70% and 25%, respectively, of that observed for cells cultured in glucose medium. In glucose cultures, where NaCl was substituted by an isotonic concentration of sorbitol, the total glycerol production and the intracellular concentration were ±10% of that in the NaCl cultures, confirming previous observations [1,8] that glycerol production and accumulation respond to osmolality rather than to specific stress solutes.

3.3. *Trehalose levels*

Only cells cultured on ethanol appreciable amounts of trehalose during exponential growth in basal medium (30.3 ± 4.4 nmol/mg dry wt.; mean ± SD of three samples of one culture). When cultured in medium containing 0.68 M NaCl, the trehalose content of the cells was 3-fold increased to 90.3 ± 8.6 nmol/mg dry wt. The raffinose grown cells produced small amounts of trehalose in 0.68 M NaCl medium (4.54 ± 1.2 nmol/mg dry wt.), whereas the concentration in cells grown in glucose was < 1 nmol/mg dry wt.

3.4. *Specific activity of GPD*

With all carbon sources, increased salinity caused an increase in the specific activity of GPD that was 2–3-fold for cells cultured at 0.34 M NaCl and to 4–6-fold for cells grown at 0.68 M NaCl (Table II). Although the increased salinity produced an increase of the enzyme activity irrespective of the carbon source, the levels of the GPD activity were clearly dependent on the source of carbon and significantly lower for cells grown on glucose than on raffinose or ethanol. When using sorbitol as the stress-solute the specific activity of GPD was increased to a level similar to that of cells grown with isoosmotic concentrations of NaCl, whereas an isotonic concentration of glycerol only caused a slight increase of the GPD activity.

3.5. *Immunoblot analysis*

Cell-free extracts of cultures grown in glucose, raffinose or ethanol medium containing various concentrations of NaCl, were subjected to SDS-PAGE and nitrocellulose blots. The immuno-complexes revealed one major protein (Fig. 2) with an apparent molecular weight of 43000 ± 1000 (mean of three determinations ± SD). This is consistent with the reported molecular weight for the *S. cerevisae* GPD in SDS-gels [22]. Densitometric scanning demonstrated a high correlation between the changes in intensity of the immunoblot and the changes of the specific activity of the enzyme. When increasing the salinity of the glucose medium the absorbance of the immunoreactive band increased 2.9-fold at 0.34 M NaCl and 5.7-fold at 0.68 M NaCl. The corresponding increases in specific activity of GPD were 2.8- and 5.2-fold, respectively (Table II).

Table II

Specific activity of GPD in cell free extracts of *S. cerevisiae* grown in media containing different carbon sources and different concentrations of stress solute

Solutes added (conc.)	Specific activity of GPD (nmol/min/mg protein)		
	Glucose	Raffinose	Ethanol
None	6.3 ± 1.6	17.8 ± 1.9	10.4 ± 1.0
NaCl (0.34 M)	17.8 ± 2.3	23.6 ± 4.1	23.6 ± 4.1
NaCl (0.68 M)	31.0 ± 6.0	66.0 ± 2.0	52.1 ± 5.9
Sorbitol (1.11 M)[a]	26.1 ± 3.2	ND	ND
Glycerol (1.11 M)[a]	12.5 ± 1.3	ND	ND

Enzyme activities given represent the mean ± SD of results obtained with homogenates from three independent cultures, each assayed at 6 different protein concentrations.

ND = not determined.

[a] Isotonic with 0.68 M NaCl.

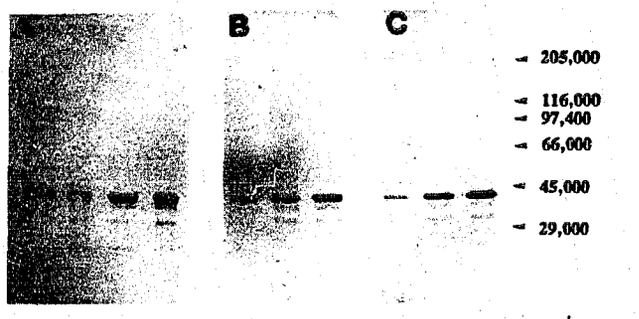

Fig. 2. Immunoblot analysis of cell free extracts of *S. cerevisiae* grown in (A) glucose-, (B) ethanol- or (C) raffinose medium containing, from left to right, 0 M, 0.34 M, 0.68 M and (for glucose medium only) 1.0 M NaCl. Within each panel all lanes were loaded with the same amount of protein, but A, B, and C are not comparable due to differences in the amount applied to each set of lanes. Molecular weight standards are indicated on the right.

4. DISUSSION

For *S. cerevisiae*, there is a growing amount of experimental data to demonstrate that glycerol production and intracellular accumulation increase in cells subjected to osmotic stress [1,6,8,9]. This response should be understood on the basis of the requirement to counter osmotic dehydration and loss of turgor/volume after exposure to increased external osmolality.

During growth on glucose, *S. cerevisiae* ferments glucose to ethanol despite aerobic conditions [11] and due to catabolite repression most mitochondrial enzymes are expressed at low levels [12–15]. Therefore, glycerol production under these conditions has a simple rationale: to maintain the cellular redox balance by serving as a sink for reducing equivalents [10,11,23]. To examine whether the osmotically induced glycerol production is specifically linked to glucose fermentation or will occur also when the cells are given increased respiratory capacity or resort to strict respiration, *S. cerevisae* was grown in media containing a carbon source that does not elicit catabolite repression (raffinose), and one which is non-fermentable (ethanol). With these substrates no or little glycerol was formed under non-stress conditions (Table I). However, at high salinity glycerol productions was induced, and glycerol was accumulated to high intracellular concentrations in cells grown in glucose and raffinose media. Assuming an intracellular soluble space of 2 μl/mg dry wt. yeast [24], the intracellular glycerol concentrations for cells grown at 0.68 M NaCl were 0.6 M in the raffinose culture and 0.9 M in the glucose culture. When adding contributions to the intracellular osmolality from amino acids [25], Na^+, K^+ [26] and basal metabolites [18], both these glycerol levels would be sufficient to generate a positive turgor pressure of the cells. Glycerol production from ethanol was induced by osmotic stress, but the production was relatively low, about 10% of that in glucose medium, and it sustained an intracellular glycerol level of about 0.2 M. This suggests that cells growing on ethanol would have to accumulate additional solutes maintain turgor under osmotic stress. GLC analysis of cell extracts revealed that such cells, beside glycerol, also accumulated trehalose during exponential growth. Although the intracellular level of this disaccharide was 3-fold increased in cells grown in saline media, the accumulated concentration gave only slight contribution to the overall intracellular osmolality (about 20% of the glycerol contribution). Thus, the means by which cells achieve sufficient turgor under these conditions remains an open question.

An additional observation worth noting relates to the proportion of the glycerol produced that was retained within the cells. In basal glucose medium all glycerol was released to the surrounding medium, whereas at high salinity cells grown in glucose, raffinose and ethanol retained approx. 15%, 20% and 30%, respectively, of the total production (Table I). These observations might be explained by postulating a pathway across the membrane which specifically increases glycerol permeability when turgor pressure surpasses a critical threshold value. Given the different roles of glycerol, such a system might act as a 'safety valve' to prevent generation of a detrimentally high turgor pressure under non-stress conditions.

Glycerol is produced in *S. cerevisiae* by a GPD catalyzed reduction of dihydroxyacetone phosphate to glycerol 3-phosphate which is then dephosphorylated by an uncharacterized phosphatase to glycerol [16]. Cells subjected to osmotic stress show an increased specific activity of GPD (Table II; [6,17]). This salinity induced increase is blocked by cycloheximide [6], and immunoblot analysis (Fig. 1) showed that the amount of GPD increased in parallel with the increased specific activity. These results strongly suggest that the induction is due to *de novo* synthesis of GPD. The GPD levels increased to similar extents regardless of whether the stress solute was NaCl or isoosmolar sorbitol (Table II), indicating regulation in response to osmolality rather than to specific solutes, similar to what is observed for the regulation of the glycerol production (see Results). However, when the external osmolality was adjusted by glycerol instead of sorbitol or NaCl, a significantly lower GPD level was observed in the cells. Since glycerol penetrates membranes more easily than the other stress solutes [27], passive influx of glycerol may lead to a diminished requirement for glycerol production to maintain the appropriate intracellular glycerol concentration. The result suggests involvement of turgor/volume in the control of GPD production. This is analogous to the lack of induction of the bacterial Kdp [28] and ProU [29] transport systems for potassium and betaine, respectively, when glycerol was used as osmoticum instead of NaCl or sucrose.

Apart from being osmotically regulated, the synthesis of GPD appears to be subject to catabolite repression as

evidenced by lower enzyme levels in glucose medium than under conditions under which such repression does not occur (Table II), such as in raffinose [30] or ethanol medium [14]. This might appear as a paradox, since the glycerol production is considerably higher in cultures grown on glucose than with the other carbon sources. However, considering that the respiratory system [14,15] and the mitochondrial glycerol 3-phosphate dehydrogenase (EC 1.1.99.5) [31] are derepressed when glucose is substituted by a non-repressing carbon source, there might be a significant competition for glycerol 3-phosphate between the phosphatase that produces glycerol and the glycerol 3-phosphate shuttle, which acts to transport reducing equivalents to the respiratory system [13]. Thus, the higher activities of GPD in cells grown in ethanol or raffinose medium might merely reflect an increased shuttle activity in these cells.

Although GPD, due to a central role in cellular metabolism is subject to regulation by several factors, it is clear that the production of this enzyme responds to osmolality and that the enzyme thus may be an eucaryotic equivalent to the osmotically regulated proteins involved in the accumulation of osmolytes in bacteria [2].

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