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Asparaginase II of *Saccharomyces cerevisiae*

GLN3/URE2 Regulation of a Periplasmic Enzyme

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ABSTRACT

The production of some extracellular enzymes is known to be negatively affected by readily metabolized nitrogen sources such as NH_4^+ although there is no consensus regarding the involved mechanisms. Asparaginase II is a periplasmic enzyme of *Saccharomyces cerevisiae* encoded by the *ASP3* gene. The enzyme activity is not found in cells grown in either ammonia, glutamine, or glutamate, but it is found in cells that have been subjected to nitrogen starvation or have been grown on a poor source of nitrogen such as proline. In this report it is shown that the formation of this enzyme is dependent upon the functional *GLN3* gene and that the response to nitrogen availability is under the control of the *URE2* gene product. In this respect the expression of ASP3 is similar to the system that regulates the *GLN1*, *GDH2*, *GAP1*, and *PUT4* genes that codes for glutamine synthetase, NAD-linked glutamate dehydrogenase, general amino-acid permease, and high affinity proline permease, respectively.

Index Entries: Asparaginase II; Nitrogen regulation; *GLN3*; *URE2*; Gene expression; *Saccharomyces cerevisiae*.

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INTRODUCTION

The production of some extracellular enzymes such as proteases and ligninases is known to be negatively affected by eadily metabolized nitrogen sources such as NH_4^+. The majority of the data argues in favor of repressive effects rather than imbalance in the metabolic pool *(1–8)*. At present there is substantial molecular data on yeast nitrogen regulation for some intracellular enzymes and permeases where the Ure2p/Gln3p transcription system plays a central role *(9)*. The aim of the present study was to investigate if this same molecular system would regulate the formation of the extracellular yeast asparaginase II in a similar fashion. From the industrial and technological point of view, the understanding of the nitrogen regulatory mechanisms of gene expression for extracellular enzymes would be a tool for process optimization. The data for the nitrogen regulation of extracellular asparaginase could be further explored to provide adequate conditions for a range of extracellular enzymes production.

Asparaginase II (L-asparagine amidohydrolase, EC 3.5.1.1) located in the pericellular space of *Saccharomyces cerevisiae* is induced by nitrogen starvation, and not by asparagine *(10)*. Growth on poor nitrogen substrates like proline results in intermediate to high enzyme activity, whereas complete nitrogen starvation for several hours in the presence of glucose results in the highest asparaginase II activity *(10,11)*. *ASP3*, which encodes asparaginase II, has been cloned and was used to demonstrate that *ASP3* mRNA was absent from ammonia grown cells and that ASP3 mRNA accumulated for several hours during nitrogen starvation *(12)*. The apparent correlation between the increase of asparaginase II activity and of *ASP3* mRNA during nitrogen starvation suggests that transcriptional control is the primary determinant of asparaginase II activity.

Two classes of mutants that affect the ability to produce asparaginase II have been described: mutants that produce high levels of asparaginase II during growth on repressing nitrogen sources such as asparagine or ammonia, and mutants that produce lower than normal amounts of asparaginase II activity during nitrogen starvation. The strongest of the derepressing class of mutants, *gdhCR* (also known as *and 4*), is known to be allelic to *ure2 (11,13,14)*. The URE2 product is a negative regulator of the transcriptional activator Gln3p such that *ure2* mutants produce high levels of mRNAs of nitrogen-regulated genes like *GLN1*, *GDH2*, *GAP1*, and *PUT4* that codes for glutamine synthetase NAD-linked glutamate dehydrogenase, general amino-acid permease and high affinity proline permease, respectively during growth on the repressing nitrogen source glutamine *(15–19)*. The strongest of the repressing class of mutants, *asp6*, was shown to be independent of the structural gene *ASP3* and produced no asparaginase II activity during nitrogen starvation *(20)*. The authors have now examined the formation of asparaginase II in mutant cells with insertions in *URE2* and/or in *GLN3* and that consequently lack the corre-

sponding protein products. By introducing plasmids carrying the *GLN3* or *URE2* genes to these mutants, it is shown that the ability of cells to form asparaginase II requires a functional *GLN3* gene and that the response to the availability of nitrogen requires a functional *URE2* gene.

MATERIALS AND METHODS

Strains and Plasmids

The *Saccharomyces cerevisiae* strains and plasmids used in this work are listed in Table 1. Yeast transformation was performed by the method of Ito et al. *(21)*. DH5α *E. coli* cells transformation and plasmid isolation were carried out as described in Sambrook et al., 1989 *(22)*.

Minimal medium consisted of 20g/L of glucose, ammonium sulfate 9.9 g/L, and yeast nitrogen base without amino acids and ammonium sulfate (Difco) 6.7 g/L. Nutritional supplements (histidine, uracil, and adenine) were added at the concentrations specified by Sherman et al. *(23)* when required. Amino acid nitrogen sources (glutamine, glutamate, or proline) were added at 1.0 g/L in place of ammonia in some experiments.

Cells were grown at 30°C on a rotary shaker at 160 rev. min^{-1}. Growth was monitored by measurement of optical density at 600 nm in a Gilson Stasar II spectrophotometer.

Asparaginase II Induction and Assay

The effects of nitrogen limitation on the formation of the enzyme were determined in the following manner *(24,25)*. Two cultures were inoculated with washed cells from an overnight culture to an OD 600 nm of 0.1. The experimental culture was grown to an OD 600 nm of 0.7–0.8 and the cells were collected by centrifugation and washed with 20 m*M* potassium phosphate buffer pH 7.0 at 4°C. Half of these cells were stored in 1–2 mL of this buffer at 4°C to serve as a prestarvation control. Derepression of the other half of the experimental culture was accomplished by incubation of the cells in phosphate buffer containing 30 g/L glucose and no source of nitrogen for 3 h. The control culture was grown without interruption and was harvested at the same time as the nitrogen starved experimental culture. In all cases, the asparaginase II levels of cells from the control cultures and the prestarvation cultures were similar, therefore, data for the control cultures are not reported here. Cells from all three growth conditions were prepared for assay of asparaginase II activity by centrifugation, washing with 20 m*M* potassium phosphate buffer pH 7.0 at 4°C, and resuspending in 1–2 mL of the same buffer. At the time of assay, the cells were diluted in the same buffer. Asparaginase II activity was assayed in intact cells to ensure that intracellular asparaginase I enzyme did not contribute to the result *(10,26)*. It was not determined whether any of the strains employed produced asparaginase I activity. L-asparagine was added to the cell suspen-

Table 1
Strains and Plasmids

Strain	Genotype	Source
Σ1278b	*MATα*	Wiame(11)
X2180-1A	*MATα, SUC2, mal, gal2, CUP1*	YGSC
P40-2a	*MATα, his4-619, leu2-3,112, ure2Δ11::LEU2*	Coschigano (15)
BMY344	*MATα, ade2-102, ura3-52, leu2-3,112, ure2Δ11::LEU2*	Coschigano (15)
BMY224	*MATα, ade2-102, ura3-52, leu2-3,112, ure2Δ11::LEU2, gln3Δ4::LEU2*	Minehart (18)
MP38	*MATα, ura3-52, leu2-3,112*	Minehart (18)
Plasmid	Markers	Source
pPM7	AatII fragment of *GLN3* cloned into AatII site of Yep24	Minehart (18)
p1-XS	XbaI-SalI fragment of *URE2* cloned into NheI-SalI site of Yep24	Coschigano (15)
pEC22	BamHI-ClaI fragment of PCR amplified ASP3 cloned into BamHI-ClaI sites of pKS^+	This work
pEC23	BamHI-SalI fragment of pEC22 ASP3 cloned into BamHI-ClaI sites of pKP15 (18)	This work

sion (OD 600 nm of 0.6) to a final concentration of 500 m*M* and this reaction mixture was incubated at 23°C. At regular intervals up 30 min, 5 mL samples were withdrawn and filtered to stop the reaction. The ammonia concentration of the supernatant was measured. It was determined that these experimental conditions allowed the measurement of the enzyme activity under initial rate conditions.

The amount of ammonia in the supernatants was measured spectrophotometrically by coupling to glutamate dehydrogenase (Boehringer-Mannheim, Indianapolis, IN). The results are presented as Δ μg/mL NH_3.

Glutamine Synthetase Induction and Assay

The nitrogen regulation of the production of glutamine synthetase was tested in cells grown in glutamine or glutamate minimal medium to midlog phase. The glutamine grown cells were harvested, washed, and resuspended in various media or starved for nitrogen. After 3 h of incubation the cells were harvested and cell extracts were prepared for the enzyme assay as previously described *(15)*. Protein concentration of the extracts was measured by the Bradford assay using reagents from Bio-Rad Laboratories (Richmond, CA). Glutamine synthetase activity was measured as previously described *(15)*.

PCR Amplification, Cloning, and ASP3 Constructed Plasmids

A pair of custom 26-mers (5'-CTGGATCCCACCAACCTCCAACTATG and 5'-AGATCGATTGGCGTACTGTGGGGCAT) were used to amplify the ASP3 gene from genomic DNA of strain P40-3C. The 1003 bp PCR product from –23 position relative to ATG and 207 pb after the stop codon (GenBank/EMBL Data Bank, Kim et al., 1988) *(13)* was cloned in the BamHI-ClaI sites of the pBlueScript KS^+ (Stratagene). The resulting plasmid was denominated pEC22. The pEC23 was constructed by transferring the ASP3 fragment from pEC22 to the BamHI-SalI sites, being fused to the CYC1-$UAS_{GAL1-GAL10}$ region of plasmid pKP15 *(18)*.

DNA Manipulation

DNA digestions with restriction enzymes, ligation, and filling in reactions were carried out as suggested by the supplier of the enzymes (BioLabs).

RESULTS

Asparaginase II Activity in Wild Type Strains

The yeast strain Σ1278b, which was previously shown to be incapable of producing asparaginase II activity *(10)*, was used as negative control. No asparagine hydrolysis was detectable in suspensions of Σ1278b after growth on ammonia minimal medium or upon nitrogen starvation for 3 h. However, suspensions of strain X2180-1A did exhibit asparagine hydrolysis after nitrogen starvation. As expected from previous studies *(13)*, X2180-1A did not produce asparaginase II activity during growth on ammonia minimal medium. Strain X2180-1A also did not produce asparaginase II activity during growth on glutamine or glutamate minimal media, but again a shift to nitrogen-free medium from either of these media resulted in the appearance of significant enzyme activity. In contrast, growth of X2180-1A on proline minimal medium did result in the appearance of enzyme activity (Table 2).

Table 2
Asparaginase II Activities of Strain X2180-1A During Growth on Various Nitrogen Sources and after Nitrogen Starvation

	Enzyme activity (Δ μg NH_3/mL)	
Nitrogen source	Pre-starvation	Post-starvation
NH3	0.1	2.4
glutamine	0.2	2.5
glutamate	0.25	4.0
proline	4.3	3.4

Table 3
Effects of Gln3p and of Ure2p on the Formation of Asparaginase II[a]

		Enzyme activity ((Δ μg NH_3/mL)	
Strain	Genotype	Pre-starvation	Post-starvation
BMY224	*gln3, ure2*	0.2	0.2
BMY224/p1-XS[b]	*gln3, URE2*	0.2	0.2
BMY224/pPM7	*GLN3, ure2*	2.7	4.3
BMY344	*GLN3, ure2*	1.7	2.2
BMY344/P1-XS	*GLN3, URE2*	0.2	1.4

[a]The cultures contained ammonium as source of nitrogen.

[b]This strain failed to produce asparaginase II when grown with proline as source of nitrogen.

Asparaginase II is Derepressed in *ure2* Strains

The product of the *URE2* gene has been shown to be required for the negative regulation of the transcriptional activator Gln3p during growth on repressing nitrogen sources such as glutamine *(15)*. A strain in which the *URE2* gene was replaced by a *ure2::LEU2* null allele also produced high levels of asparaginase II activity during growth on ammonia (Table 3). When this strain was transformed with the *URE2* plasmid p1-XS, asparaginase II activity was not produced during growth on ammonia but was produced by nitrogen starvation.

Similar experiments were performed with strain BMY224 that carries the disrupted null alleles *ure2::LEU2* and *gln3::LEU2* (Table 3). Both BMY224 and BMY224 transformed with the *URE2* plasmid pl-XS were incapable of producing asparaginase II activity during nitrogen starvation. Very high asparaginase II activity was obtained during growth on ammonia and during nitrogen starvation in BMY224 transformed with the *GLN3* plasmid pPM7. These results are entirely consistent with those obtained in the study of the transcriptional regulation of the *GLN1* and *GDH2* genes *(15,16,18)*, indicating that the *ASP3* gene is transcriptionally regulated by the products of *URE2* and *GLN3*. In accord with previous studies, *gln3* is epistatic to *ure2* with respect to the production of asparaginase II activity and asparaginase II production is unregulated by nitrogen source in a *ure2 GLN3* strain *(15,27)*. It appears that the product of *URE2* is required to block transcriptional activation of *ASP3* by the product of *GLN3*.

Glutamine Synthetase is Derepressed by Nitrogen Starvation

The effects of nitrogen starvation on another gene that responds to activation by Gln3p were tested. Glutamine synthetase (GS), a product of *GLN1*, is found at low levels in a wild type strain during growth on glutamine and at approx 30-fold higher levels during growth on glutamate, whereas in a *gln3* strain the difference in GS levels between glutamate- and glutamine-grown cells is only threefold *(15)*. In the present work the level of GS of nitrogen-starved cells was compared to glutamine- or glutamate-grown cells. After 3 h of nitrogen starvation, the GS activity increased approx 20-fold compared to glutamine-grown cells (Table 4). Glutamate-grown cells produced about fourfold higher GS activity than nitrogen-starved cells. In order to directly compare the relative strength of cells grown on glutamate vs nitrogen starvation as signals for the production of GS, cells were shifted at midlog phase from minimal glutamine medium to either glutamine, glutamate, or nitrogen starvation medium for 3 h. GS activity increased about 20-fold in the nitrogen starved cells and about 39-fold in the cells shifted to glutamate medium, whereas no signifcant difference was observed in cells shifted from glutamine to glutamine (Table 4). GS levels are highest during growth on glutamate and intermediate after nitrogen starvation, whereas asparaginase II levels are highest after nitrogen starvation and are low during growth on glutamate. Thus, there appears to be a difference in the production of GS and asparaginase II in response to the same nitrogen signals and by the same transcriptional activator, Gln3p.

An Attempt to Overproduce Asparaginase II

Despite some strains, like BMY 344, show high levels of asparaginase II as compared to other strains (Table 3), we aimed to manipulate the ASP3 gene in order to increase enzyme production. The pEC23 was used to

Table 4
Glutamine Synthetase Activity of Strain X2180-1A During Growth on Glutamine or Glutamate and after Shift from Glutamine to other Media

Nitrogen source	Shifted to[a]	Specific activity (μmole/mg.min^{-1})
glutamate	–	2.43
glutamine	–	0.03
glutamine	glutamine	0.02
glutamine	glutamate	1.34
glutamine	no nitrogen	0.60

[a]Cells were collected by filtration, washed with water, resuspended in the indicated medium, and incubated under normal growth conditions for 3 h.

transform MP38, BMY224, and BMY344 strains. The selected transformants showed no asparaginase II activity related to the presence of the plasmid under the described conditions. This subject is currently under investigation to clear up these results.

DISCUSSION

Using a molecular approach it has been shown in this report that the mechanism of nitrogen control of the production of asparaginase II activity is mediated by the Ure2p/Gln3p transcriptional system. Interestingly, the pattern of nitrogen control on *ASP3* appears to be somewhat different than that on *GLN1*, which also responds to Ure2p/Gln3p. *GLN1* is most highly transcribed and GS activity is the highest when the nitrogen source is glutamate and GS activity is intermediate during nitrogen starvation. However, *ASP3* transcription during growth in glutamate (as judged by the production of asparaginase II activity) appears to be moderate *(11)* to low (Table 2). The disparity between the levels of GS and asparaginase II is even more striking if glutamate- vs proline-grown cells are compared. The amount of GS was sixfold higher in glutamate- than in proline-grown Σ1278b cells *(27)*, compared to 16-fold less asparaginase II in glutamate- than in proline-grown X2180-1A cells (Table 2). The possibility that the Ure2p/Gln3p transcription system produces different effects on different promoters under same nitrogen conditions is intriguing. Whether or not this effect is transcriptional must be clarified first because it is conceivable that asparaginase II activity or the secretion of asparaginase II is regulated posttranscriptionally by the nitrogen source, making it impossible to infer the transcriptional state of *ASP3* from asparaginase II activity.

It has been understood for some time that this asparaginase hydrolase is secreted into the pericellular space when the nitrogen source is poor or absent, and that growth on asparagine represses the production of asparaginase II activity *(10)*. This is the opposite of the expected pattern of regulation: high asparaginase II activity in the presence of high concentrations of asparagine. One possibility is that the intracellular asparaginase I, whose production does not appear to be regulated by nitrogen source, is present in sufficient quantities to allow good growth on high concentrations of asparagine, but that growth is limited by poor asparagine transport when the asparagine concentration is low. If the rate of ammonia or aspartate transport is higher than the rate of transport of equivalent, low concentrations of asparagine, then extracellular asparagine hydrolysis could compensate for poor transport. Another possibility is that nitrogen starvation allows *GLN3* to activate the production of a wide range of secreted enzymes. Interestingly, the primary nitrogen regulator of *Neurospora crassa*, the product of the *nit-2* gene and its analogous *areA* gene in *Aspergillus nidulans* encodes a metal finger domain homologous to that of the GLN3 protein *(28,29)*. In that respect similar patterns of nitrogen regulation in yeast and fungi could be envisaged. The use of yeast cells to study the subject is a tool to characterize the relevant genes and regulatory systems. These data have a potential use to engineer strains for increased extracellular enzymes production. Also, the evaluation of the effects of the nitrogen source on extracellular asparaginase II would allow the use of a scientific approach for fermentation medium design with obvious economic advantages, mainly taking into account the scale of extracellular enzymes production.

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