Repression of nitrogen catabolic genes by ammonia and glutamine in nitrogen-limited continuous cultures of *Saccharomyces cerevisiae*

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Growth of *Saccharomyces cerevisiae* on ammonia and glutamine decreases the expression of many nitrogen catabolic genes to low levels. To discriminate between ammonia- and glutamine-driven repression of *GAP1*, *PUT4*, *GDH1* and *GLN1*, a *gln1-37* mutant was used. This mutant is not able to convert ammonia into glutamine. Glutamine-limited continuous cultures were used to completely derepress the expression of *GAP1*, *PUT4*, *GDH1* and *GLN1*. Following an ammonia pulse, the expression of *GAP1*, *PUT4* and *GDH1* decreased while the intracellular glutamine concentration remained constant, both in the cytoplasm and in the vacuole. Therefore, it was concluded that ammonia causes gene repression independent of the intracellular glutamine concentration. The expression of *GLN1* was not decreased by an ammonia pulse but solely by a glutamine pulse. Analysis of the mRNA levels of *ILV5* and *HIS4* showed that the response of the two biosynthetic genes, *GDH1* and *GLN1*, to ammonia and glutamine in the wild-type and *gln1-37* was not due to changes in general transcription of biosynthetic genes. *Ure2p* has been shown to be an essential element for nitrogen-regulated gene expression. Deletion of *URE2* in the *gln1-37* background prevented repression of gene expression by ammonia, showing that the ammonia-induced repression is not caused by a general stress response but represents a specific signal for nitrogen catabolite regulation.

Keywords: nitrogen regulation, ammonia, glutamine, *Saccharomyces cerevisiae*

INTRODUCTION

Saccharomyces cerevisiae is able to use a wide variety of nitrogen sources for growth. Growth on medium containing ammonia or glutamine results in relatively fast growth compared to growth on medium containing proline or urea. Consequently, ammonia and glutamine are classified as good nitrogen sources, in contrast to proline and urea. Ammonia may be used as sole nitrogen source. Ammonia and α -ketoglutarate are converted into glutamate by NADPH-dependent glutamate dehydrogenase (NADPH-GDH). Subsequently glutamate and ammonia are converted into glutamine by glutamine

synthetase (GS). Glutamate and glutamine are used as amino donors in all other biosynthetic reactions (Cooper, 1982; Magasanik, 1992; Wiame *et al.*, 1985). Glutamine can also be used as sole nitrogen source. Intracellular glutamine is used to produce glutamate by several enzymes. The enzyme glutamate synthase converts one molecule of glutamine and one molecule of α -ketoglutarate into two molecules of glutamate (Cogoni *et al.*, 1995; Roon *et al.*, 1974). Moreover, glutaminases have been identified in yeast which degrade glutamine into glutamate and ammonia; the latter is used again by NADPH-GDH to produce glutamate (Soberón & González, 1987a, b).

During growth on good nitrogen sources, the activities of enzymes involved in the utilization of poor nitrogen sources are decreased. This phenomenon is called

Abbreviations: GDH, glutamate dehydrogenase; GS, glutamine synthetase.

nitrogen catabolite repression (Cooper, 1982; Magasanik, 1992; Wiame *et al.*, 1985). One type of regulation of these enzyme activities occurs at the level of gene expression. During growth on glutamine the transcription of, for example *GAP1* and *PUT4*, encoding the general amino acid permease and the proline permease, respectively, is repressed (Jauniaux *et al.*, 1987; Jauniaux & Grenson, 1990). It has been suggested that glutamine activates Ure2p, a protein having homology with glutathione *S*-transferases, which in turn inactivates a transcription activator, Gln3p (Blinder & Magasanik, 1995; Coschigano & Magasanik, 1991; Courchesne & Magasanik, 1988; Minehart & Magasanik, 1991). The decreased activity of Gln3p then causes decreased transcription of *GAP1* and *PUT4*. It has been suggested that Gln3p is inactivated by Ure2p via modification of thiol groups on Gln3p (Coschigano & Magasanik, 1991; Magasanik, 1992). Results have also been published which indicate that Ure2p acts directly on Gln3p to block its ability to activate transcription (Blinder *et al.*, 1996). In addition to Gln3p, another positive regulator, designated Gat1p/Nil1p, participates in the transcription of genes sensitive to nitrogen catabolite repression (Coffman *et al.*, 1996; Stanbrough *et al.*, 1995). Growth on ammonia leads to high intracellular glutamine concentrations and it is generally believed that this leads to repression of gene expression (Mitchell & Magasanik, 1984).

Gln3p is required for expression of the transcription of a wide variety of genes involved in nitrogen metabolism, including *UGA1*, *CAN1*, *DAL4*, *DAL1*, *DAL2*, *DAL7*, *GDH2*, *GLN1* and *GDH1* (Daugherty *et al.*, 1993). Also, the transcription of vacuolar protease genes *CPS1*, *PEP4*, *PRB1* and *LAP4* is positively regulated by Gln3p (Coffman & Cooper, 1997). The Gln3p-regulated genes *GDH2*, *GLN1* and *GDH1* encode NAD-dependent GDH (NAD-GDH), GS and NADPH-GDH, respectively (Benjamin *et al.*, 1989; Miller & Magasanik, 1992; Minehart & Magasanik, 1992; Mitchell, 1985; Nagasu & Hall, 1985). NAD-GDH degrades glutamate into α -ketoglutarate and ammonia. During growth on glutamate, the amount of NAD-GDH is high due to high-level expression of the *GDH2* gene. High intracellular glutamate concentrations may stimulate Gln3p and consequently induce the transcription of Gln3p-regulated genes (Courchesne & Magasanik, 1988). The transcriptional regulation of *GLN1* is more complex. In addition to Gln3p regulation, *GLN1* is also activated by Gcn4p, a transcription factor whose activity increases under amino acid starvation conditions due to an enhanced translation of *GCN4* mRNA (Dever *et al.*, 1992). Therefore, the expression of *GDH2* and *GLN1* may differ depending upon the growth conditions. The expression of *GDH1* is regulated by the ammonia concentration in the medium (ter Schure *et al.*, 1995b) and by Leu3p, a transcriptional regulator in the biosynthesis of branched-chain amino acids, which activates *GDH1* transcription (Hu *et al.*, 1995).

Although the pathways underlying the regulation of nitrogen metabolism are now known, little is known

about the role of key metabolites as stimulators or inhibitors of these pathways. In ammonia-limited continuous cultures it has been shown that the expression of *GAP1* decreases when the extracellular ammonia concentration is increased (ter Schure *et al.*, 1995a). In contrast, the intracellular glutamine concentration remained constant. However, the ammonia flux and growth rate also changed and could have caused the repression. In continuous cultures having a constant ammonia flux and growth rate, it has been shown that *GAP1* and *PUT4* expression is regulated in response to the extracellular ammonia concentration (ter Schure *et al.*, 1995b). Whereas under these conditions the growth rate and ammonia flux do not change, the intracellular glutamine concentration increases with increasing ammonia concentration. Therefore, the repressed transcription could be caused by both the intracellular glutamine concentration as well as the ammonia concentration.

To discriminate between ammonia- and glutamine-induced signals regulating nitrogen metabolism, a *gln1-37* strain (Mitchell & Magasanik, 1983) which is unable to synthesize glutamine was used. Immunoprecipitation experiments showed that the *gln1-37* mutant produced an amber-suppressible truncated Gln1p product (Mitchell & Magasanik, 1983). This strain was grown in a glutamine-limited continuous culture and pulsed with either ammonia or glutamine. The glutamine limitation caused complete derepression of the *GAP1* and *PUT4* genes. When ammonia was added to the culture of the *gln1-37* mutant, no change was observed in the intracellular glutamine concentration; however, the mRNA levels of *GAP1* and *PUT4* decreased rapidly. Thus, ammonia represses *GAP1* and *PUT4* expression independently of the intracellular glutamine concentration. In previous studies it has been shown that addition of ammonia to *gln1-37* cells grown in glutamine-limited continuous cultures represses enzyme activity of arginase but not of NAD-dependent glutamate dehydrogenase (Dubois *et al.*, 1977). There was only partial repression of urea amidolyase activity. This repression could be exerted at the level of transcription, translation or enzyme activity. The data presented in this study show that ammonia repression operates at the level of transcription. A *gln1-37* mutant also lacking *URE2* did not show this ammonia-specific repression of *GAP1* and *PUT4* expression, showing that *URE2* is essential for ammonia-specific repression of transcription.

METHODS

Strains and growth conditions. The following *S. cerevisiae* strains were used: Σ 1278b (wild-type), *gln1-37* (*gln1*) and U37 (*gln1 ure2*). Σ 1278b and *gln1-37* were kindly provided by Professor Dr B. Magasanik (MIT, Cambridge, MA, USA). A *ura3* mutation was introduced in *gln1-37* by gene replacement of *URA3* with a linear disruption fragment and subsequent selection on agar plates containing 5-fluoro-orotic acid (Sigma). Strain U37 was constructed according to the method of Wach *et al.* (1994) by replacement of *URE2* in the *gln1 ura3* background with a linear DNA fragment containing the

complete *URA3* gene flanked by two regions, containing 181 and 200 bp, respectively, from the 5' and 3' prime ends of *URE2*. The disruption fragment was constructed by PCR using the following primers: (1) 5' GCCGAGCTCCTCGAG-GTTGAAAAGAATAGC, (2) 5' GCAATGCGATAGCGG-GATCCTTTGGTGTACAACCTTAATTTG, (3) 5' GGAT-CCCGCTATCGCATTGCGTTTACAAATGGACGAAGC and (4) 5' GCCCTGCAGGAATTCTGTGGTTGGGG. The 181 bp fragment was synthesized using primers 1 and 2; the 200 bp fragment was synthesized using primers 3 and 4. These two fragments were used as template in one PCR using primers 1 and 4. The synthesized fragment was cloned as a *Pst*I/*Sac*I fragment into pUC19. *URA3* was inserted into the constructed *Bam*HI site of the vector. The linearized vector was used as DNA fragment for disruption of *URE2*. Disruption was checked by performing PCR on the replaced chromosomal locus.

Batch cultures were grown in minimal medium (Yeast Nitrogen Base without amino acids; Difco) containing 2% glucose. When glutamine was used as nitrogen source, cells were grown in minimal medium (Yeast Nitrogen Base without amino acids and ammonium sulphate; Difco) supplemented with 2% glucose and 0.3% glutamine. During continuous culture experiments, the strains were grown at 30 °C in a 2 l BiofloIII fermenter (New Brunswick Scientific) connected to a computer controller unit running Advanced Fermentation Software (New Brunswick Scientific). The strains were inoculated in a medium described by Sierkstra *et al.* (1992) except that 3 g glutamine l⁻¹ was used instead of ammonia as sole nitrogen source. After overnight growth, a continuous feed containing the same medium composition was connected. The glucose concentration of the medium was 20 g l⁻¹. Before and during the pulse experiments, the glutamine concentration in the feed was 3 g l⁻¹. During batch and continuous growth, the pH was automatically controlled at 5 by the addition of 5 M KOH. The airflow was 1.5 l min⁻¹ and the O₂ tension was kept above 50%. CO₂ production [r_{CO_2} in mmol h⁻¹ (g dry wt)⁻¹] and O₂ consumption [q_{O_2} in mmol h⁻¹ (g dry wt)⁻¹] were measured on-line by connection of the headspace of the fermenter to a Uras3G CO₂ analyser and a Magnos4G O₂ analyser (Hartmann and Braun).

Determination of glutamine-limited growth. The wild-type Σ 1278b and the mutant *gln1-37* strains were grown in continuous culture using glutamine as sole nitrogen source and glucose as sole carbon source. To determine at which concentration glutamine was limiting, the concentration was gradually increased in steps of 0.025 g l⁻¹ starting at 1 g l⁻¹. Glucose was used as carbon source at 20 g l⁻¹ and the concentrations of all other components, including glucose, remained constant. The dilution rate was set to 0.1 h⁻¹. At each steady-state the biomass concentration was measured. Up to 3 g glutamine l⁻¹ the biomass increased linearly with the glutamine concentration (results not shown), indicating that glutamine was limiting. Concentrations higher than 4 g glutamine l⁻¹ caused no further increase in biomass, indicating that glutamine was not limiting anymore and glucose had become limiting.

To ensure glutamine limitation, both the *gln1-37* mutant and the corresponding wild-type Σ 1278b were grown with 20 g glucose l⁻¹ and 3 g glutamine l⁻¹ in the feed at a dilution rate of 0.1 h⁻¹.

Pulsing of ammonia and glutamine. Ammonia or glutamine were pulsed into the culture by rapidly adding (within 10 s) 40 ml of a sterilized concentrated stock solution of either NH₄Cl or glutamine to final concentrations of 40 and 20 mM, respectively.

Sampling and preparation of samples. During glutamine-limited growth, steady-state samples were taken as described previously (ter Schure *et al.*, 1995a). After pulsing, samples were taken at time intervals as indicated. Samples were collected for determination of biomass concentration, metabolite concentration and mRNA levels. Biomass analyses and preparation of cell-free extracts for the determination of amino acids were performed as described previously (ter Schure *et al.*, 1995a).

Total RNA was isolated by washing the cells with 1 ml extraction buffer (1 mM EDTA, 100 mM LiCl, 100 mM Tris/HCl, pH 7.5, 10 mM iodoacetic acid). The cell pellet was resuspended in 1 ml extraction buffer in a glass tube and an equal volume of glass beads was added. After vortexing, 100 μ l 10% SDS and 1.5 ml phenol were added. Cells were lysed by vortexing six times for 30 s each time. The cell suspension was centrifuged for 15 min at 4 °C and the remaining supernatant was extracted three times with phenol/chloroform (1:1). Thereafter, potassium acetate was added to a final concentration of 2% and RNA was precipitated using 2.5 ml 96% ethanol. The RNA was used in Northern blotting analyses.

For the isolation of intracellular α -ketoglutarate, glutamate and glutamine after the glutamine pulses, 1 ml culture sample was quickly filtered using a Whatman GF/C filter and the filter was then washed with 5 ml H₂O at 4 °C. The filter containing the yeast pellet was frozen in liquid nitrogen. α -Ketoglutarate was isolated by adding 20 ml boiling ethanol to 5 ml frozen culture sample. The extract was heated at 100 °C for 5 min and then concentrated to 5 ml by evaporation. Cytosolic and vacuolar amino acid pools were isolated according to the method of Ohsumi *et al.* (1988).

Determination of metabolites and free amino acids. Extracellular metabolites and free amino acid concentrations were analysed as described previously (ter Schure *et al.*, 1995a). α -Ketoglutarate was determined by drying 200 μ l cell-free ethanol extract using vacuum evaporation. The remaining pellet was resuspended in 1 ml reaction mixture. α -Ketoglutarate was determined enzymically as described by Bergmeyer *et al.* (1974) using 50 mM imidazole, pH 7.5, 200 mM KCl, 2 mM EDTA and 20 mM CaCl₂ as buffer.

Northern blotting analysis. For the detection of *GAP1*, *PUT4*, *GLN1*, *GDH1*, *ILV5*, *HIS4* and *ACT1* mRNAs, labelled oligonucleotides were used as described previously (Sierkstra *et al.*, 1992; ter Schure *et al.*, 1995b). Labelling of oligonucleotides and Northern blotting analyses were performed as described previously (Sierkstra *et al.*, 1992). Northern blots were stripped by boiling in 0.1% SDS, according to the manufacturer's instructions, and reprobed with *ACT1* as internal control for the amount of mRNA blotted. Hybridization was carried out as described by Sambrook *et al.* (1989). Northern blots were quantified by densitometry on a Personal Densitometer SI. Different exposure times were used to obtain reliable densitometry data. All scanning data were corrected for the *ACT1* level measured in each lane. Data were quantified by calculating the intensity ratio between the entire band of the gene of interest and of the reference gene. All values are given as the mean of at least three independent samples.

RESULTS

GAP1, *PUT4*, *GDH1* and *GLN1* are expressed during glutamine-limited growth

To discriminate between ammonia- and glutamine-induced repression of *GAP1*, *PUT4*, *GDH1* and *GLN1*,

Table 1. Expression of *GAP1*, *PUT4*, *GDH1* and *GLN1* mRNAs in wild-type ($\Sigma 1278b$) and mutant cells (*gln1-37*)

$\Sigma 1278b$ and *gln1-37* were grown under the culture conditions indicated. The data were quantified by calculating the intensity ratio between the band of the gene of interest and of the reference gene *ACT1*. The *GAP1*, *PUT4*, *GDH1* and *GLN1* intensity ratios of the wild-type under batch culture with proline were set arbitrarily at 100%. The expression levels under other conditions are presented as percentages of this level of expression.

Strain	Culture conditions	Level of gene expression (%)			
		<i>GAP1</i>	<i>PUT4</i>	<i>GDH1</i>	<i>GLN1</i>
Wild-type	Batch culture with glutamine	20	11	38	36
Wild-type	Continuous, glutamine-limited	98	108	100	88
<i>gln1-37</i>	Continuous, glutamine-limited	104	104	70	179
Wild-type	Batch culture with proline	100	100	100	100

Fig. 1. Ammonia and glutamine concentrations in the culture medium of wild-type $\Sigma 1278b$ ($\blacklozenge$) and mutant *gln1-37* ($\blacksquare$) grown in glutamine-limited continuous cultures. When steady-state was reached, ammonia or glutamine was added to the culture medium. (a) Extracellular ammonia concentration after the addition of 40 mM NH_4Cl to glutamine-limited cells. (b) Extracellular glutamine concentration after the addition of 15 mM glutamine to glutamine-limited cells. All values are the mean of three independent samples; the SD did not exceed 5%. See Methods for details.

the *gln1-37* mutant was used. Mitchell & Magasanik (1983) have shown that this mutant produces a truncated glutamine synthetase protein, which is due to an amber mutation. Because of the amber mutation, full length *GLN1* mRNA can still be detected in the mutant, making it functional for studying *GLN1* expression. Growth of the mutant in batch cultures on glutamine leads to repression of *GAP1*, *PUT4*, *GDH1* and *GLN1*

(ter Schure *et al.*, 1995a) and hence it cannot be used to study ammonia repression. To investigate if glutamine-limited growth will lead to derepression of *GAP1*, *PUT4*, *GDH1* and *GLN1*, as is the case during ammonia-limited growth (ter Schure *et al.*, 1995b), strain *gln1-37* and the corresponding wild-type strain, $\Sigma 1278b$, were grown in glutamine-limited continuous cultures as described in Methods.

As shown in Table 1, *GAP1*, *PUT4*, *GDH1* and *GLN1* are derepressed in both the wild-type and the mutant during glutamine-limited growth, whereas these genes were repressed during batch growth of $\Sigma 1278b$ on glutamine. This is consistent with the notion that during glutamine limitation the intracellular concentration of glutamine is not high enough to cause repression. The intracellular glutamine content was determined to be approximately $10 \mu\text{mol g}^{-1}$ in both wild-type and mutant and the residual glutamine concentration in the culture medium was 0.7 mM for both.

The expression levels of *GAP1*, *PUT4*, *GDH1* and *GLN1* during glutamine-limited growth were compared with the expression levels of these genes during growth in batch cultures with proline as sole nitrogen source. As shown in Table 1, the expression of *GAP1*, *PUT4*, *GDH1* and *GLN1* are fully derepressed compared to growth in batch cultures with proline. The *GLN1* mRNA level in *gln1-37* was higher compared to that in the wild-type. However, it is not known whether the stability of *gln1-37* mRNA is equal to or greater than that of wild-type *GLN1* mRNA.

Effect of ammonia on the expression of *GAP1*, *PUT4*, *GDH1* and *GLN1*

To investigate the influence of ammonia on the expression of the genes, *gln1-37* and $\Sigma 1278b$ were grown in glutamine-limited continuous cultures and after steady-state was reached, ammonia was added to the culture medium of both strains as described in Methods. As shown in Fig. 1(a), the NH_4^+ concentration in the culture medium increased from zero before the pulse to about 40 mM immediately after the pulse for both strains.

Fig. 2. Expression of (a, e) *GAP1*, (b, f) *PUT4*, (c, g) *GDH1* and (d, h) *GLN1* mRNAs in wild-type $\Sigma 1278b$ ($\blacklozenge$) and mutant *gln1-37* ($\blacksquare$) cells grown in glutamine-limited continuous cultures after the addition of 40 mM NH_4Cl (a, b, c, d) or 15 mM glutamine (e, f, g, h) to the culture medium. The data were quantified by calculating the intensity ratio between the band of the gene of interest and of the reference gene, *ACT1*. For each gene the intensity ratio at steady-state glutamine-limited growth ($t = 0$) was 100%. The expression levels at the other time points are presented as percentages of expression at $t = 0$. See Methods for details.

Thereafter, the NH_4^+ concentration in the medium decreased due to uptake of ammonia by the yeast on one hand and dilution of ammonia by the continuous addition of fresh medium without ammonia to the culture on the other.

To examine mRNA expression of nitrogen-regulated genes, total mRNA was isolated from both wild-type and mutant strains at different times after addition of

ammonia. The samples taken just before the NH_4^+ pulse were marked as $t = 0$ and succeeding samples were taken at various time intervals after addition of ammonia as indicated. The expression of *GAP1*, *PUT4*, *GDH1* and *GLN1* mRNAs was analysed using Northern blotting analysis as described in Methods. After adding ammonia to the wild-type culture, the mRNA level of *GAP1* as well as that of *PUT4* decreased (Fig. 2a, b). Ten minutes after the addition of ammonia, no *GAP1* mRNA

Fig. 3. Amounts of intracellular glutamine and glutamate in wild-type $\Sigma 1278b$ (◆) and mutant *gln1-37* (■) cells after the addition of 40 mM NH_4Cl (a, c) or 15 mM glutamine (b, d) to the culture medium. (a, b) Intracellular glutamine levels after the addition of 40 mM NH_4Cl (a) or 15 mM glutamine (b) to glutamine-limited cells. (c, d) Intracellular glutamate levels after the addition of 40 mM NH_4Cl (c) or 15 mM glutamine (d) to glutamine-limited cells. All values are the mean of three independent samples; the SD did not exceed 5%. See Methods for details.

could be detected. After addition of ammonia to the *gln1-37* culture, the *GAP1* and *PUT4* mRNA levels also decreased. Again, after 10 min *GAP1* and *PUT4* mRNAs were undetectable.

In agreement with previous studies (ter Schure *et al.*, 1995b) the ammonia pulse to the wild-type culture also resulted in repression of *GDH1* (Fig. 2c). The fact that this was also the case in the *gln1-37* mutant demonstrates that this repression is not due to its conversion to glutamine.

Magasanik and coworkers (Miller & Magasanik, 1992; Minehart & Magasanik, 1992) have shown that the expression of *GDH2* and *GLN1* is repressed during growth on glutamine. The expression of *GLN1* mRNA was not repressed by the ammonia pulse to the mutant (Fig. 2d) in contrast to the wild-type. After addition of ammonia to the wild-type culture, the level of *GLN1* mRNA decreased. Because ammonia causes an increase in intracellular glutamine concentration, it probably represses *GLN1* transcription via an increase in the intracellular glutamine concentration.

Intracellular glutamine and glutamate content after addition of ammonia

To investigate whether the observed repression was caused by increased ammonia concentrations or indirectly by an increase in the intracellular glutamine concentration, free amino acids were isolated and the intracellular glutamine content was analysed.

As shown in Fig. 3(a), the intracellular glutamine content increased from 10 to 60 $\mu\text{mol g}^{-1}$ after 120 min in wild-type cells. However, the intracellular glutamine concentration remained constant at 10 $\mu\text{mol g}^{-1}$ in the *gln1-37* mutant. Together with the Northern data presented in Fig. 2, this demonstrates that ammonia represses transcription independently from an increase in the total intracellular glutamine concentration.

GAP1 expression is activated by the transcriptional regulator Nif1p during growth on glutamate (Stanbrough *et al.*, 1995). A decrease in the intracellular glutamate concentration after the ammonia pulse might have caused the observed decrease in *GAP1* expression. Therefore, the intracellular glutamate content after the ammonia pulse was analysed. As shown in Fig. 3(c), in both the wild-type and the mutant strains the glutamate concentration decreased from 42 and 70 $\mu\text{mol g}^{-1}$ to 20 and 55 $\mu\text{mol g}^{-1}$, respectively, 10 min after the addition of ammonia. Thereafter, in both the mutant and the wild-type, the glutamate concentration increased again and reached its original steady-state level at 30 min after the pulse. The observed changes in intracellular amino acid contents were not caused by a change in the biomass concentration.

In *gln1-37* the glutamate level is at least 10 $\mu\text{mol g}^{-1}$ higher than in the wild-type at $t = 0$, even at its lowest concentration, while in the wild-type *GAP1*, *PUT4* and *GDH1* expression is derepressed at zero time (Fig. 2). Therefore, the observed ammonia repression is not caused by changes in intracellular glutamate concentration.

Thus, ammonia is able to repress the transcription of *GAP1*, *PUT4* and *GDH1* independently from its conversion to glutamine or glutamate.

During conditions of nitrogen limitation, yeast cells start to accumulate amino acids in their vacuoles. Batch growth on various nitrogen sources results in different distributions of amino acids between the vacuole and the cytosol. Hence, addition of ammonia to *gln1-37* cells might result in a change in the concentrations of glutamate or glutamine within these two compartments and indirectly trigger gene repression. To determine whether the addition of ammonia to glutamine-limited cells resulted in a redistribution of glutamine, cytosolic and vacuolar amino acids pools were isolated according to the method of Ohsumi *et al.* (1988). During glutamine-limited growth, about 57% of total glutamate is present in the cytosol while the remaining 43% is present in the vacuole (data not shown). Of the total glutamine pool, 30% is present in the cytosol and 70% in the vacuole. Within 30 min of the addition of ammonia to glutamine-limited *gln1-37* cells no change in the distribution of glutamate was observed (data not shown). Since complete repression of *GAP1*, *PUT4* and *GDH1* expression was observed within 10 min after the addition of ammonia (Fig. 2), the effect of ammonia on gene expression is not caused by a redistribution of glutamate or glutamine between cytosol and vacuole.

Effect of glutamine pulse on the expression of *GAP1*, *PUT4*, *GDH1* and *GLN1*

To examine the glutamine-induced repression of *GAP1*, *PUT4*, *GDH1* and *GLN1*, glutamine was pulsed to the culture medium of both wild-type $\Sigma 1278b$ and the mutant *gln1-37*. As shown in Fig. 1(b), the glutamine concentration in the culture medium of both the wild-type and the mutant increased from about 0.7 mM just before the pulse to about 15 mM directly after the pulse.

The mRNA levels of *GAP1*, *PUT4*, *GDH1* and *GLN1* decreased after addition of glutamine to both wild-type and *gln1-37* mutant cells (Fig. 2e, f, g, h). The decrease in *GLN1* mRNA expression in *gln1-37* was not observed after an ammonia pulse.

Intracellular glutamine and glutamate contents after addition of glutamine

The changes in the intracellular glutamine and glutamate contents were analysed after glutamine pulses to both the wild-type and *gln1-37* strains to compare the data with those from the ammonia pulse. As shown in Fig. 3(b), following addition of glutamine to *gln1-37* cells, the intracellular glutamine concentration increased rapidly to about 325 $\mu\text{mol g}^{-1}$ within 20 min and remained constant up to 60 min after the pulse. This was also observed when glutamine was added to the wild-type strain; however, the maximum level observed in the wild-type was about 230 $\mu\text{mol g}^{-1}$. In both the mutant and wild-type, the intracellular glutamate concentration remained constant at about 50 and 90 $\mu\text{mol g}^{-1}$, re-

Fig. 4. Expression of *GAP1* mRNA in *gln1-37* cells in response to ammonia after different times of nitrogen starvation. *gln1-37* cells were grown in glutamine-limited continuous culture. After steady-state was reached, the cells were transferred to nitrogen starvation medium and incubated at 30 °C for the time indicated. Ammonia (40 mM) was then added to the nitrogen-starved cells and 20 min after the addition of ammonia the cells were harvested and analysed for *GAP1* mRNA expression using Northern blotting analysis. The data were quantified by calculating the intensity ratio between the band of the gene of interest and of the reference gene, *ACT1*. The intensity ratio at steady-state glutamine-limited growth was 100%. The expression levels at the other time points are presented as percentages of expression during steady-state glutamine-limited growth. See Methods for details.

spectively (Fig. 3d). Thus, addition of glutamine to glutamine-limited cells did not change the intracellular glutamate concentration.

Ammonia repression of *GAP1* is blocked during nitrogen starvation

Previous experiments have shown that temperature-sensitive *gln1* mutants (*gln1^{ts}*) do not repress *GAP1* expression when incubated in medium containing ammonia as sole nitrogen source. However, from the above data it is clear that glutamine-limited *gln1-37* cells do repress *GAP1* expression upon addition of ammonia and this is not due to increased intracellular glutamine concentrations. The difference between ammonia-incubated *gln1^{ts}* cells and the glutamine-limited *gln1-37* cells is that the *gln1^{ts}* cells are starved for glutamine and do not grow, whereas the *gln1-37* cells are continuously fed with low concentrations of glutamine and do grow. Therefore, the ammonia repression seems to be modulated by starvation conditions.

To examine the effect of nitrogen starvation on ammonia repression, *gln1-37* cells, pregrown in glutamine-limited continuous culture, were starved for nitrogen for different periods of time, after which ammonia was added to the cells. During glutamine-limited growth, *GAP1* expression is derepressed (Fig. 2). When glutamine-limited cells were directly incubated for 10 min in ammonia-containing medium, repression of *GAP1* expression was observed ($t = 0$). The same result was observed when these cells were starved for nitrogen up to 10 min prior to incubation with ammonia (Fig. 4). However, when the cells were starved for nitrogen for

Fig. 5. Expression of (a) *HIS4* and (b) *ILV5* mRNAs in wild-type $\Sigma 1278b$ (◆) and mutant *gln1-37* (■) cells grown in glutamine-limited continuous cultures after the addition of 40 mM NH₄Cl to the culture medium. The data were quantified by calculating the intensity ratio between the band of the gene of interest and of the reference gene, *ACT1*. For each gene the intensity ratio at steady-state glutamine-limited growth ($t = 0$) was 100%. The expression levels at the other time points are presented as percentages of expression at $t = 0$. See Methods for details.

more than 15 min, the addition of ammonia did not result in the repression of *GAP1* expression. These results show that nitrogen starvation blocks the ability of ammonia to repress *GAP1* expression. Addition of glutamine to *gln1^{ts}* cells growing at the restrictive temperature relieves nitrogen starvation and hence induces *GAP1* repression.

Ammonia and glutamine repression of *GDH1* and *GLN1* is not due to a change in the general regulation of transcription of biosynthetic genes

The expression of *GDH1* and *GLN1* is controlled by nitrogen sources via Ure2p and Gln3p, two proteins regulating the expression of genes involved in nitrogen catabolism. *GDH1* and *GLN1* play a role in the biosynthesis of glutamate and glutamine, which are the amino donors in all other biosynthetic reactions. It could well be that the observed expression patterns of these two genes is caused by changes in the overall transcription rate of genes involved in amino acid synthesis. Such general transcription regulators are Leu3p or Gcn4p in the case of *GDH1* and *GLN1*, respectively (Dever *et al.*, 1992; Hu *et al.*, 1995). As transcription of amino acid biosynthetic genes is stimulated in response to starvation for any one of several amino acids (Hinnebusch, 1988) it could be expected

that relief of glutamine limitation by addition of ammonia would lead to a decrease in transcription of amino acid biosynthetic genes.

To investigate if the transcription of other genes involved in amino acid synthesis was influenced by ammonia or glutamine, the mRNA levels of *HIS4* and *ILV5* were analysed. In contrast to expectation, the expression of *HIS4* and *ILV5* both increased after the addition of ammonia to *gln1-37* cells (Fig. 5a, b). Similar results were obtained when ammonia was pulsed to the wild-type cells (Fig. 5a, b). Thus, the expression of *HIS4* and *ILV5* increased, whereas the expression of *GDH1* and *GLN1* decreased after addition of ammonia or glutamine. Therefore, the observed repression of *GDH1* and *GLN1* expression is not a reflection of the general decreased expression of genes involved in amino acid biosynthesis.

URE2 is involved in the repression of *GAP1*, *PUT4* and *GDH1* by ammonia

Deletion of *URE2* leads to derepression of NAD-GDH and GS during growth on glutamine as shown by Courchesne & Magasanik (1988). To examine the role of *URE2* in ammonia-specific transcriptional repression, this gene was replaced by the *URA3* gene in the *gln1-37* background as described in Methods. The *gln1 ure2* double mutant was designated U37. Addition of ammonia to U37 grown at steady-state in glutamine-limited continuous culture would demonstrate whether ammonia repression is nitrogen-catabolite-regulated or whether another mechanism is involved.

Strain U37 was grown in a glutamine-limited continuous culture using the same growth conditions as for $\Sigma 1278b$ and *gln1-37*. The growth parameters during steady-state were identical except that the biomass concentration was 8 instead of 9 g l⁻¹. After steady-state was reached, 40 mM ammonia was added to the culture medium. Total RNA was isolated and the amounts of *GAP1*, *PUT4* and *GDH1* mRNAs were analysed. As shown in Fig. 6, the *GAP1* and *PUT4* mRNA levels initially decreased to about 50% at 10 min after the addition of ammonia. However, after 10 min, the *GAP1* and *PUT4* expression levels increased again to about 75 and 100%, respectively, of the levels during glutamine-limited growth.

In contrast to the *gln1 ure2* double mutant, the expression of *GAP1* and *PUT4* was almost completely repressed after the addition of ammonia to the *gln1* single mutant (Fig. 2). This demonstrates that *URE2* is involved in ammonia-specific repression of *GAP1* and *PUT4*.

After the ammonia pulse to the U37 culture, the level of *GDH1* and *GLN1* mRNAs increased. This is in accordance with the hypothesis that in a *ure2* background the repression mechanism is eliminated and only the activation mechanism, reflected by *ILV5* and *HIS4* expression, is active.

Fig. 6. Expression of (a) *GAP1*, (b) *PUT4*, (c) *GDH1* and (d) *GLN1* mRNAs in strain U37 grown in glutamine-limited continuous culture after the addition of 40 mM NH_4Cl to the culture medium. The data were quantified by calculating the intensity ratio between the band of the gene of interest and of the reference gene, *ACT1*. For each gene the intensity ratio at steady-state glutamine-limited growth ($t = 0$) was 100%. The expression levels at the other time points are presented as percentages of expression at $t = 0$. See Methods for details.

The involvement of Ure2p in the ammonia-specific repression demonstrates that ammonia repression is a nitrogen catabolite repression-specific signal.

DISCUSSION

When glutamine or ammonia is used as sole nitrogen source in batch cultures of yeast cells, the expression of *GAP1*, *PUT4* and *GDH1* is repressed. This repression is thought to be caused by high intracellular glutamine concentrations. However, previous studies have indicated a specific role of ammonia in this regulation of gene repression (ter Schure *et al.*, 1995a, b). To examine whether ammonia must be converted into glutamine to effect gene repression, the *gln1-37* mutant was grown in a glutamine-limited continuous culture and subsequently ammonia or glutamine was pulsed into the culture. The *gln1-37* mutation, described by Mitchell & Magasanik (1983), has been shown to generate a truncated GS protein which lacks activity.

During steady-state glutamine-limited growth, no physiological differences were observed between the wild-type $\Sigma 1278b$ and *gln1-37*. This demonstrated that the wild-type and the mutant used glutamine equally well as nitrogen source. Only the intracellular glutamate concentration differed. Also, after pulsing ammonia to both $\Sigma 1278b$ and *gln1-37*, no significant physiological differences were observed between wild-type and mutant strains.

After adding ammonia to *gln1-37* culture, as well as to $\Sigma 1278b$, the expression of *GAP1*, *PUT4* and *GDH1* mRNAs decreased, indicating that ammonia is able to repress transcription. No increase in intracellular glutamine, neither in the cytosol nor in the vacuole, was observed. This showed that ammonia generates repression independently of the intracellular glutamine concentration. This repression of gene expression is not due to diminished ATP production or lowered growth rates and neither is the repression of *GDH1* due to a decrease in the general expression of biosynthetic genes.

These data show that metabolism of ammonia to glutamine or glutamate is not required to trigger nitrogen catabolite repression at the level of transcription. In addition, the partitioning data indicate that ammonia does not induce a redistribution of either glutamate or glutamine between cytosol and vacuole. However, it cannot be excluded that ammonia is metabolized in some unknown signalling molecule which might trigger the response. The addition of glutamine also leads to repression of transcription. Intracellular glutamine can be degraded to glutamate and ammonia by glutaminases (Soberón & González, 1987a, b).

To prove that repression after addition of glutamine is due to glutamine itself or its conversion to ammonia, the intracellular ammonia concentration should be determined. However, ammonia is a weak base and will diffuse across the membrane quite fast. Therefore, *in*

vitro measurement of the ammonia concentration will not reveal the actual intracellular concentration, e.g. washing the cells will shift the equilibrium between the intra- and extracellular ammonia concentration. One solution to this problem would be *in vivo* measurement of intracellular ammonia with non-invasive techniques, e.g. NMR, but attempts to carry out such studies have not been successful.

At this moment the intracellular ammonia concentration can only be estimated. Recent studies in our laboratory have led to the development of a metabolic computer model of the experiments described in this study (van Riel *et al.*, 1998). This model predicted an intracellular ammonia concentration of about 200 $\mu\text{mol (g dry wt)}^{-1}$ during steady-state glutamine-limited growth. After the addition of glutamine, only a slow increase in the intracellular ammonia concentration is predicted, resulting in a two-fold higher ammonia concentration 2 h after the pulse.

Earlier studies revealed repression of arginase activity and partial repression of urea amidolyase activity, but not of NAD-GDH, by the addition of ammonia to *gln1-37* cells grown in glutamine-limited continuous culture (Dubois *et al.*, 1977). This repression could be exerted at the level of transcription, translation or enzyme activity. The repression of *GAP1* and *PUT4* mRNAs by ammonia demonstrates that ammonia repression operates at the level of transcription. The Ure2p protein regulates nitrogen repression via Gln3p at the level of transcription (Blinder *et al.*, 1996). Addition of ammonia to a *gln1-37* mutant with a *URE2* deletion demonstrated that *URE2* is essential for this ammonia-specific pathway.

The specific repression of gene expression by ammonia has a number of implications. First, there has to be a sensor for ammonia concentration, either intracellular or extracellular. We have shown previously that it is in fact the ammonia concentration which is sensed (ter Schure *et al.*, 1995b). Putative sensors might be Mep1p or Mep2p, identified as ammonia transporters by Marini *et al.* (1994).

Second, a signalling pathway must exist which transduces the signal from the sensor to the transcription apparatus. The lack of repression by ammonia in strain U37 showed that Ure2p is one component of this pathway. However, besides Ure2p, another repression mechanism has to be active to explain the initial decrease in *GAP1* and *PUT4* mRNAs in the *ure2 gln1* double mutant. Coffman *et al.* (1995) have shown a *URE2*-independent repression pathway which could explain residual repression after addition of ammonia to *ure2 gln1* cells. However, addition of ammonia to *ure2 gln1* cells only led to a transient repression. Ure2p is essential for repression of transcription via inactivation of the transcription factor Gln3p. Therefore, Gln3p is also likely to be a component of the pathway. In addition, it has been shown that Uga43p/Dal80p is also negatively regulated by Ure2p (André *et al.*, 1995). Thus, the involvement of Ure2p in ammonia-specific repression

implies that ammonia repression is a more general repression signal than just for Gln3p-regulated genes.

Third, a group of genes exists which is repressed by ammonia as well as glutamine, e.g. *GAP1*, *PUT4* and *GDH1*, but *GLN1* is solely repressed by glutamine. This means that the yeast cell is able to differentiate between glutamine- and ammonia-derived signals. In both cases Ure2p is involved. However, it should be taken into account that the equilibria between NH_4^+ and glutamine which exist in a wild-type cell will not be reached in *gln1-37* cells lacking GS. This will inevitably influence purine synthesis. Distinct from the *GDH1*, *GAP1* and *PUT4* promoter, the *GLN1* promoter contains a purine-responsive element which might be responsible for the increased *GLN1* expression after addition of ammonia to *gln1-37* cells. Therefore, care should be taken in concluding that glutamine represses *GLN1* expression directly.

It has already been demonstrated that inactivation of Gap1p by ammonia can be separated from inactivation by glutamate or glutamine in a *per1* mutant (Courchesne & Magasanik, 1988). Moreover, a regulatory mechanism involving dephosphorylation and possibly ubiquitination and degradation of Gap1p by ammonia has been described (Hein *et al.*, 1995). It might well be that the same ammonia-sensing protein is directing two signals, one to the *GAP1* protein to initiate rapid inactivation and degradation of the transporter and another towards the transcription machinery to prevent further synthesis of the protein.

The finding of Wickner (1994) that the *ure3* mutation is a prion analogue of Ure2p and our finding that Ure2p is involved in ammonia-specific repression could indicate a mechanism in which a change in conformation of Ure2p in response to intracellular ammonia concentrations is involved. Studies in our laboratory are in progress to establish the components and mechanisms underlying the ammonia-sensing pathway.

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