Essential Role of One-carbon Metabolism and Gcn4p and Bas1p Transcriptional Regulators during Adaptation to Anaerobic Growth of Saccharomyces cerevisiae

Bonny M. Tsoi, Anthony G. Beckhouse, Cristy L. Gelling, Mark J. Raftery, Joyce Chiu, Abraham M. Tsoi, Lars Lauterbach, Peter J. Rogers, Vincent J. Higgins, and Ian W. Dawes

From the School of Biomedical and Health Sciences, University of Western Sydney, Penrith, New South Wales and the Ramaciotti Centre for Gene Function Analysis, School of Biotechnology and Biomolecular Science, and the Bioanalytical Mass Spectrometry Facilities, University of New South Wales, Sydney 2052, and Foster’s Group Limited, 4-6 Southampton Crescent, Abbotsford 3067, Australia

The transcriptional activator Gcn4p is considered the master regulator of amino acid metabolism in Saccharomyces cerevisiae and is required for the transcriptional response to amino acid starvation. Here it is shown that Gcn4p plays a previously undescribed role in regulating adaptation to anaerobic growth. A gcn4 mutant exhibited a highly extended lag phase after a shift to anaerobiosis that was the result of L-serine depletion. In addition, the one-carbon metabolism and purine biosynthesis transcriptional regulator Bas1p were strictly required for anaerobic growth on minimal medium, and this was similarly due to L-serine limitation in bas1 mutants. The induction of one-carbon metabolism during anaerobiosis is needed to increase the supply of L-serine from the glycine and threonine pathways. Using a number of experimental approaches, we demonstrate that these transcription regulators play vital roles in regulating L-serine biosynthesis in the face of increased demand during adaptation to anaerobiosis. This increased L-serine requirement is most likely due to anaerobic remodeling of the cell wall, involving de novo synthesis of a large number of very serine-rich mannoproteins and an increase in the total serine content of the cell wall. During anaerobic starvation for L-serine, this essential amino acid is preferentially directed to the cell wall, indicating the existence of a regulatory mechanism to balance competing cellular demands.

Saccharomyces cerevisiae can grow rapidly aerobically and anaerobically, and this has led to its use in the study of oxygen sensing and the requirement of molecular oxygen for metabolism. Under anaerobic conditions cells cannot synthesize ste- rol and unsaturated fatty acids because the two pathways require molecular oxygen (1). Several studies have identified the genome-wide transcriptional responses of yeast growing in aerobic or anaerobic conditions (2–6), however, many environments are subject to dynamic fluctuations in oxygen tension and, hence, there is current interest in how organisms respond to changes in oxygen level (3, 7, 8). Under anaerobic conditions proteins involved in amino acid metabolism are synthesized at higher levels (9). We have previously reported that cells lacking the Gcn4p transcription factor regulating genes involved in amino acid metabolism have a growth defect under anaerobic conditions. This indicates that there may be an altered requirement for some Gcn4p-dependent aspect of amino acid biosynthesis in anaerobic cells compared with aerobic (10).

In S. cerevisiae and related fungi, including Candida albicans, Gcn4p is a master transcriptional activator that directly activates over 30 amino acid biosynthetic genes for biosynthesis of 19 amino acids, as well as regulating many other cellular processes, including purine biosynthesis, organelle biosynthesis, autophagy, glycogen homeostasis, and stress responses (11). The mechanisms involved in Gcn4p regulation have been reviewed by Hinnebusch (12). When cells are subjected to stresses such as amino acid, purine, or nitrogen limitation, Gcn4p expression is translationally induced via a pathway that depends on the GCN2-encoded protein kinase, and there are human homologues of genes in the Gcn4p regulation pathway, including GCN2 (13, 14).

During this work one-carbon metabolism was implicated in the cellular response to a shift to anaerobiosis. One-carbon metabolism includes the reactions whereby one-carbon units are transferred from the donors L-serine, glycine, or formate via tetrahydrofolate (H4folate)4 derivatives to essential biosyn- thetic processes, including methyl group biogenesis and the synthesis of nucleotides, vitamins, and some amino acids (15, 16). In many organisms, serine is the main one-carbon donor, and its conversion to glycine via the cytoplasmic serine hydroxymethyltransferase (encoded in S. cerevisiae by SHM2) leads to the formation of the key intermediate 5,10-methylene tetrahydrofolate (5,10-CH2-H4folate). Serine can also be formed from glycine and 5,10-CH2-H4folate by reversal of this

* This work was supported by Linkage and Discovery Grants from the Australian Research Council (to I. W. D., V. J. H., and P. J. R.) and by Australian Research Council Postgraduate Awards (to B. M. T., C. L. G., A. M. T., and A. G. B.).

§ The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1 and S2 and Tables S1–S3.

1 Present address: Eskitis Institute for Cell and Molecular Therapies, Griffith University, Brisbane 4111, Australia.

2 Present address: Institute of Biology/Microbiology, Humboldt-Universität zu Berlin, Chausseestrasse 117, Berlin 10115, Germany.

3 To whom correspondence should be addressed. Tel.: 61-2-9385-2089; Fax: 61-2-9385-1050; E-mail: i.dawes@unsw.edu.au.

4 The abbreviations used are: H4folate, tetrahydrofolate; GCRE, general control-responsive element; 5,10-CH2-H4folate, 5,10-methylene tetrahydrofolate; SD, synthetic defined medium; MS, mass spectrometry; MS/MS, tandem MS.
Adaptation to Anaerobiosis in S. cerevisiae

reaction. Glycine is a one-carbon donor via the action of the strictly mitochondrial glycine decarboxylase complex.

The interconversion of one-carbon donors and intermediates is highly regulated at the level of enzyme activity as well as at transcription (17, 18). In S. cerevisiae, many of the genes involved form a regulon that responds to the cytoplasmic level of 5,10-CH₂-15Hfolate (16, 19, 20). The transcription factor Bas1p, which regulates purine biosynthesis (21), is also involved in regulating expression of the genes of the one-carbon regulon (22, 23). The aim of this study was to identify the role played by the Gcn4p transcription factor in the adaptation of cells to anaerobiosis, and to determine which processes regulated by Gcn4p were involved. The phenotype of the mutant lacking Gcn4p was found to result from an altered cellular requirement for l-serine and/or glycine when cells adapt to anaerobic conditions. Therefore the aim was extended to explore the link between one-carbon metabolism and the adaptation of cells to anaerobic conditions. The results have shown a remarkable shift in metabolic networks occurs as cells adjust to anaerobic conditions.

EXPERIMENTAL PROCEDURES

Yeast Strain—Strains used in this study are listed in Table 1. Diploid deletion strains homozygous for the relevant gene deletion were obtained from Euroscarf (Frankfurt, Germany).

Yeast Media and Aerobic Growth Conditions—S. cerevisiae was grown in YEPD containing 2% (w/v) glucose, 2% (w/v) Bacto-peptone, and 1% yeast extract; in synthetic defined (SD) medium containing 2% (w/v) glucose, 0.17% yeast nitrogen base (Difco, Franklin Lakes, NJ), or in SDC medium, which was SD supplemented with adenine (10 mg/liter), l-arginine (50 mg/liter), l-aspartate (80 mg/liter), l-histidine (20 mg/liter), l-isoleucine (50 mg/liter), l-leucine (100 mg/liter), l-lysine (50 mg/liter), l-methionine (20 mg/liter), l-phenylalanine (50 mg/liter), l-threonine (100 mg/liter), l-tryptophan (100 mg/liter), l-tyrosine (50 mg/liter), uracil (20 mg/liter), and l-valine (140 mg/liter) (24), or in SCC, which was SDC with the addition of l-alanine, l-asparagine, l-cysteine, l-glutamine, l-glutamate, glycine, l-proline, and l-serine (76 mg/liter). Where indicated formate was supplemented by adding 50 mM sodium formate. For solid media, 2% (w/v) agar was added. Cultures were incubated at 30 °C with shaking at 250 rpm. Where selection for antibiotic resistance was required 200 mg/liter Geneticin (G418, Sigma) or 100 mg/liter nourseothricin (NatMX4) by transforming each haploid with the p4339 plasmid (pCRII-TOPO::natMX4) (28) and selecting on cloNAT. The MATa deletion mutants harboring the NatMX resistance gene were then crossed with MATa deletion mutants containing the Geneticin resistance marker (kanMX). Resultant heterozygous diploids were selected from media containing both antibiotics, sporulated, and dissected to form haploid meiotic spore progeny. The genotype of each derived strain was confirmed using PCR. The confirmed MATa and MATa haploid double mutants were then crossed to generate homozygous diploid double mutants.

Assay for β-Galactosidase Activity—Specific activity of β-galactosidase in extracts of cells was assayed as described previously (29). Cell protein in the extract was measured using bovine serum albumin (Sigma-Aldrich) as a standard and Protein Assay dye reagent (Bio-Rad) as per the manufacturer’s protocol. Specific activity was expressed as nanomoles of O-nitropheryl-β-d-galactopyranoside (Sigma-Aldrich) hydrolyzed per min per µg of total protein.

Amino Acid Analysis—An overnight culture was inoculated into a 250-ml anaerobic bottle with 100 ml of medium to an A₆₀₀ of 0.2. Cultures were harvested immediately (time 0), at 15, 30, 60, 120, 240, and 360 min after inoculation, and the A₆₀₀ was measured. Upon harvesting, 50 ml of culture was filtered through a glass fiber pre-filter (Sartorius) and immediately washed with 50 ml of chilled (4 °C) SD medium without amino acids. The pre-filter was immersed in 30 ml of cold 5% (v/v) perchloric acid for 5 min to lyse the cells, and the lysate was adjusted to pH 7.0 with cold 5 M potassium hydroxide. The volume of the lysate was recorded, and 10 ml of the supernatant and 1 ml of the medium filtrate were stored for subsequent analysis.

Amino acid composition of each sample from cell lysate (2 ml) or diluted growth medium (900 µl) was analyzed using a modified high-performance liquid chromatography method.
TABE 1

| Strain     | Genotype                          | Source            |
|------------|-----------------------------------|-------------------|
| BY473      | MATa/LEU2/his3A/1-leu2AΔ/1-leu2Δ0/lys2AΔ0/lyg2AΔ0/lys2Δ0/lys2X2Δ0/MET15/uro3Δ0/uro3Δ0 | Euroscarf         |
| BY474      | MATa/LEU2/lys2AΔ0/MET15/uro3Δ0  | Euroscarf         |
| BY474      | MATα his3Δ1/1-leu2Δ0/lys2Δ0/LYS2 MET15/met13Δ0/uro3Δ0/uro3Δ0 | Euroscarf         |
| BY474      | gcn4Δ                            | Euroscarf         |
| BY474      | gcn4Δ                            | Euroscarf         |
| BY474      | bas1Δ                            | Euroscarf         |
| BY474      | gly1Δ                            | Euroscarf         |
| BY474      | ser1Δ                            | Euroscarf         |
| BY474      | tirΔ                             | Euroscarf         |
| BY474      | shm1Δ                            | Euroscarf         |
| BY474      | shm2Δ                            | Euroscarf         |
| BY474      | SHM2::lacZ                       | This study        |
| BY474      | gcn4Δ::SHM2::lacZ                 | This study        |
| BY474      | bas1Δ::SHM2::lacZ                 | This study        |
| BY474      | GCRC::lacZ                       | This study        |
| BY474      | gly1Δ::GCRC::lacZ                 | This study        |
| BY474      | bas1Δ::GCRC::lacZ                 | This study        |
| BY474      | GLY1::lacZ                       | This study        |
| BY474      | gcn4Δ::GLY1::lacZ                 | This study        |
| BY474      | bas1Δ::GLY1::lacZ                 | This study        |
| BY474      | shm1Δ::gluA                      | This study        |
| BY474      | shm1Δ::shlA                      | This study        |
| BY474      | gcn4Δ::shm1Δ::shlA                | This study        |
| BY474      | shm1Δ::shm2Δ                     | This study        |

Pico-TAG amino acid analysis, Waters, Milford, MA). Amino acids were quantified by measuring peak area in comparison to standard curves obtained using pure standards.

Isolation of Cell Walls—Cell walls were isolated from anaerobically grown cells by the method of Yin et al. (30).

Mass Spectrometric Analysis of Cell Walls—Cell walls were digested proteolytically with a modification of the method of Yin et al. (30) using the estimate that cell wall proteins accounted for ~2% of the cell wall dry mass. Freeze-dried cell walls (2 mg) were resuspended in 500 μl of 100 mM NH₄HCO₃ containing 10 mM dithiothreitol, and incubated for 1 h at 56 °C.

After centrifugation (1500 × g, 5 min), proteins in the pellet were 5-alkylated in 500 μl of 100 mM NH₄HCO₃ containing 55 mM iodoacetamide for 45 min at room temperature in the dark. Cell walls were washed three times with 100 μl of 50 mM NH₄HCO₃ and dried under vacuum. Cell walls were resuspended in 50 mM NH₄HCO₃ and digested with trypsin (using a cell wall protein to enzyme ratio of 50:1) overnight at 37 °C. Because digestion with trypsin did not reveal significant differences between aerobic and anaerobic cell walls, the samples were digested further with AspN overnight at 37 °C.

Digested samples were centrifuged, and the soluble peptides separated by nano-LC using an Ultimate 3000 high-performance liquid chromatography and autosampler system (Dionex, Amsterdam, Netherlands). Samples (2.5 μl) were concentrated and desalted onto a micro C18 precolumn (500 μm × 2 mm, Michrom Bioreources, Auburn, CA) with H₂O:CH₃CN (98:2, 0.05% heptfluorobutyric acid) at 20 μl/min. After a 4-min wash the pre-column was switched (Valco 10 port valve, Dionex) in line with a fritless nano column (75 μm x ~10 cm) containing C18 media (5 μm, 200-Å Magic, Michrom) manufactured according to Gatlin (31). Peptides were eluted using a linear gradient of H₂O:CH₃CN (98:2, 0.1% formic acid) to H₂O:CH₃CN (65:35, 0.1% formic acid) at 250 nl/min over 30 min. High voltage (1800 V) was applied to a low volume tee, and the column tip was positioned ~0.5 cm from the heated capillary (T = 200 °C) of a LTQ FT Ultra (Thermo Electron, Bremen, Germany) mass spectrometer. Positive ions were generated by electrospray, and the LTQ FT Ultra was operated in data-dependent acquisition mode. A survey scan m/z 350–1750 was acquired in the Fourier transform ion cyclotron resonance cell (resolution = 100,000 at m/z 400, with an accumulation target value of 1,000,000 ions). Up to seven of the most abundant ions (>2500 counts) with charge states of +2 or +3 were sequentially isolated and fragmented within the linear ion trap using collision-induced dissociation with an activation of q = 0.25 and activation time of 30 ms at a target value of 30,000 ions. m/z ratios selected for MS/MS were dynamically excluded for 30 s.

Peak lists were generated using Mascot Daemon/extract_msn (Matrix Science, London, United Kingdom) using the default parameters and submitted to the data base search program Mascot (version 2.2, Matrix Science). Search parameters were: Precursor tolerance, 4 ppm, and product ion tolerances, ±0.4 Da; Met(O) and Cys-carboxy-amidomethylation specified as variable modification, enzyme specificity was trypsin, AspN, 2 missed cleavages were possible, and the NCBI*nl data base (January, 2008) was searched. Scaffold (version Scaffold-2_00_01, Proteome Software Inc., Portland, OR) was used to validate MS/MS-based peptide, protein identifications, and estimate relative protein abundances.

Cell Labeling and Fractionation—Precultures grown aerobically in SDC medium were used to inoculate aerobic and anaerobic SD medium to an A₆₀₀ of 0.2. To each culture (100 ml) was added [l-¹H]serine (370 Bq/ml) and l-serine to 7.4 mM. Aliquots were harvested at 5 and 24 h after addition of label, and samples were taken to estimate total label in the culture. For each sample cells were harvested by centrifugation (3000 × g, 5 min), and washed with 10 mM Tris-HCl, pH 7.5. Wet weights of the samples were recorded prior to resuspension in 10 mM Tris-HCl, pH 7.5. Cell suspensions were divided into three portions for isolation of cell walls, total cell lipids, and for cell fractionation by differential centrifugation.

Cell walls were prepared from the pellet as indicated above. The supernatant from cell wall isolation was combined with the
first wash of the cell wall fraction and centrifuged at 15,000 × g for 30 min at 4 °C, the pellet was retained, and the supernatant was centrifuged at 100,000 × g for 1 h at 4 °C. Total cell lipid was isolated from 30 ml of culture at 5 × 10^6 cells/ml as described in a previous study (32). Radioactivity in the cell wall, total lipid, 15,000 × g pellet, and the 100,000 × g pellet and supernatant was determined by liquid scintillation counting.

RESULTS

A Strain Lacking GCN4 Exhibits an Extended Lag Phase during Anaerobiosis—Many amino acid biosynthetic genes are upregulated under anaerobic conditions (9). Because these genes are subject to control by the Gcn4p transcription factor, we previously tried to determine whether deletion of GCN4 had any effect on the regulation of gene expression during the shift from anaerobic to aerobic conditions. This led to the very surprising result that, although the gcn4Δ strain could grow as well as the wild type aerobically in defined SDC medium, it was unable to grow within 24 h following inoculation into anaerobic medium of identical composition, whereas the wild-type strain grew normally after a 5-h lag (10).

To test whether the gcn4Δ strain was unable to grow anaerobically or its growth was delayed, cells were inoculated from an aerobic culture grown in SDC medium into anaerobic SDC medium, and growth was monitored over a 50-h period. The wild-type (BY4743) strain (Fig. 1) adapted to the anaerobic environment and began exponential growth after 5 h, reaching stationary phase after 15 h. A tir3Δ-negative control strain (lacking a serine-alanine-rich cell wall mannanprotein essential for anaerobic growth) did not grow. Interestingly, the gcn4Δ strain was able to grow in the anaerobic medium, but exhibited an extended lag phase lasting 24 h after inoculation with stationary phase not reached until 45 h post-inoculation. The viability of gcn4 cells was similar to that of the wild type over 48 h (data not shown). The eventual recovery and growth of the gcn4 culture was not due to the formation of suppressor mutants, because isolated clones from 48-h anaerobic cultures also showed the extended lag phase when re-transferred from aerobic to anaerobic media. Therefore, GCN4 plays a role in the ability of cells to adapt to anaerobic growth.

The gcn2 deletion mutant that lacks the ability to initiate the translation of Gcn4p (33) was tested, because Gcn4p can be activated by different pathways. This mutant also had an extensive lag phase when inoculated into anaerobic medium, confirming that the role of GCN4 in anaerobic growth reflects the activity of the classic general control pathway. These results were not due to an involvement of Gcn4p in the uptake of auxotrophic requirements (uracil, l-histidine, and l-leucine) by the BY4743 strain, because gcn4Δ and gcn2Δ strains in the prototrophic S228C background also showed an extended delay in anaerobic growth relative to that of the wild type (Table 2).

1-Serine or Glycine Addition Markedly Reduced the Extended Lag Phase of the Gcn4 Mutant during Anaerobiosis—The SDC anaerobic medium used above did not contain l-alanine, l-asparagine, l-cysteine, l-glutamate, l-glutamine, glycine, l-proline, or l-serine (24). To determine whether any of these amino acids were essential for gcn4 cells to show wild type anaerobic growth, SDC medium individually supplemented with each of the amino acids was inoculated with the wild-type and gcn4 mutant, and growth was determined after the shift to anaerobiosis.

Supplementation with l-serine or glycine markedly shortened the adaptation delay of the gcn4 mutant (Table 3). l-Cysteine only slightly reduced the delay and addition of any other amino acid did not affect the delay at all. l-Serine and glycine can both act as donors in one-carbon metabolism, and because formate can also act as a one-carbon donor (17) the effect of adding it on the ability of cells to adapt to anaerobiosis was determined. Formate had a similar effect to l-serine in reducing the lag of the gcn4 mutant (Table 3).

These results indicate that Gcn4p is required for adaptation to anaerobiosis, because it mediates a transcriptional response.

![Figure 1. Growth of cells following transfer to anaerobic conditions for the wild-type strain BY4743 (●), gcn4 mutant (○), and tir3 mutant (□) in SDC medium. Data represent the average of biological triplicates of A_{600} with standard deviation as shown.](image-url)
to serine or glycine deficiency during the adaptation period. If so, supplementation of a wild-type culture with the deficient amino acid should also suppress induction of Gcn4p activity under anaerobic conditions.

Gcn4p Activity Increases after the Shift to Anaerobiosis in the Absence of L-Serine—Gcn4p activity during adaptation to anaerobic conditions was examined by transforming the wild-type and gcn4 mutant strain with the GCRE::lacZ fusion construct that acts as a reporter of Gcn4p activity. Specific activity of β-galactosidase was determined as an indicator of Gcn4p activity in cells shifted from aerobic to anaerobic conditions. As predicted, in the wild-type Gcn4p activity increased after the shift to anaerobic conditions, attaining a 5-fold induction by 24 h (Fig. 2). This induction of Gcn4p activity was suppressed by the addition to the medium of either L-serine or formate, but not glycine. This is consistent with the shift to anaerobiosis causing a deficiency of L-serine that induces Gcn4p translation and hence activation of the Gcn4p regulon.

Mutants Affected in Biosynthesis of L-Serine or Glycine Display a Similar Phenotype to the Gcn4 Mutant during Anaerobiosis—To determine which Gcn4p target pathways are needed for adaptation to anaerobiosis, 50 mutants affected in amino acid biosynthesis were tested under anaerobic conditions (supplemental Table S1). Of the auxotrophic strains tested, the majority displayed a phenotype similar to that of the wild type, but those with deletions of SER1 (encoding 3-phosphoserine aminotransferase), GLY1 (encoding L-threonine aldolase), or CYS3 (cystathionine β-lyase) showed similar phenotypes to that of the gcn4 mutant, although the cys3 mutant was impaired in its growth rate as well (Table 2). Fig. 3 illustrates the roles of these and related genes in the pathways for L-serine, glycine, and L-cysteine biosynthesis. The ser1 mutant had the most extreme phenotype, because it was unable to grow at all anaerobically, which differed from the gcn4 mutant. Interestingly, anaerobic growth was also delayed when L-serine or glycine was supplemented in the medium of the ser1Δ strain and when glycine (but not L-serine) was supplemented to the gly1Δ strain (data not shown). These results confirm that up-regulation of SER1 and/or GLY1 (and possibly CYS3) by Gcn4p may be required for adaptation to anaerobiosis.

The One-carbon Metabolism Regulator Bas1p Is Required for Anaerobic Growth on Minimal Medium—The Bas1p transcription factor binds the same DNA motif as...
Gcn4p (TGACTC) and is a known regulator of genes involved in one-carbon metabolism and purine biosynthesis (34). Therefore the anaerobic growth of a bas1 mutant was examined. The bas1 mutant did not grow at all after the shift to anaerobic conditions, even in the presence of adenine (which is required for aerobic growth) in the SDC medium (Fig. 4). This growth defect was rescued by supplementing SDC with either L-serine or formate but not glycine.

Bas1p Activity Increases during the Shift to Anaerobic Conditions—We hypothesized that BAS1 is required for anaerobic growth because Bas1p up-regulates genes involved in the synthesis of L-serine from one-carbon units. A key step in this conversion is catalyzed by serine hydroxymethyltransferase converting glycine and one-carbon units to L-serine. Under aerobic conditions the SHM2 gene (encoding the cytoplasmic serine hydroxymethyltransferase) is regulated by Bas1p and probably also by Gcn4p (16, 26, 27). The wild-type, bas1, and gcn4 strains were transformed with an SHM2::lacZ reporter, and expression of β-galactosidase was determined in each strain shifted to anaerobic SDC and SDC supplemented with glycine, L-serine, or formate.

The SHM2::lacZ reporter did not show detectable activity in the absence of Bas1p, confirming that it is essential for SHM2 expression (Fig. 5). In the wild-type, SHM2 expression was induced 4- to 5-fold within 5 h of the shift to anaerobic conditions and remained high even after growth had ceased. In the gcn4 mutant there was an 8-h delay before SHM2 expression was activated, but then it increased to even higher levels than in the wild type. When L-serine or formate was added there was only a small change in SHM2 expression in all strains. In the presence of glycine, SHM2 expression in the wild type increased 3-fold only after growth had ceased, but in the gcn4 mutant there was a marked increase soon after inoculation that continued throughout growth.

In summary, activation of SHM2 transcription by Bas1p in minimal medium increased on a shift to anaerobic conditions. This increase was suppressed by L-serine and formate and to a lesser extent by glycine.

Serine Hydroxymethyltransferase Activity Is Required for Anaerobic Growth on Minimal Medium—Although SHM2 is induced under anaerobic conditions, an shm2 mutant had a wild-type anaerobic adaptation time (supplemental Table S1). However, the cell also has a mitochondrial serine hydroxymethyltransferase encoded by SHM1. Therefore the growth of individual shm1 and shm2 mutants, and a double shm1 shm2 mutant (in which one-carbon metabolism can only be supported by the glycine cleavage reaction), was examined. In unsupplemented medium only the single shm1 and shm2 mutants grew normally anaerobically, the gcn4 mutant was delayed as before, but the gcn4 shm1, gcn4 shm2, and shm1 shm2 double mutants did not grow at all (data not shown). This indicates that in minimal medium at least one of the serine hydroxymethyltransferases is necessary for anaerobic growth, and in the absence of either Gcn4p needs to be active.

This additional requirement for L-serine under anaerobic growth conditions occurs despite the potential for L-serine biosynthesis from glycolysis via 3-phosphoglycerate in the SER1
Adaptation to Anaerobiosis in S. cerevisiae

pathway. These results indicate that, under anaerobic conditions, cell growth requires the provision of L-serine from one-carbon metabolism. Thus the genetic evidence shows that the direction of one-carbon metabolic flux is reversed from that in aerobic S. cerevisiae cells, which use L-serine formed from 3-phosphoglycerate as the main one-carbon donor. Under anaerobic conditions, it appears that glycine must be converted to L-serine (at least during the adaptation phase). Hence the gly1 mutant, which lacks the ability to make glycine from L-threonine, is unable to adapt to anaerobiosis in minimal medium.

Taken together with the dramatic differences in growth adaptation times when L-serine biosynthesis or one-carbon metabolism is disrupted, these results provide evidence that cells growing in anaerobic conditions require additional capacity to synthesize L-serine that is satisfied by glycine and one-carbon metabolism, and that the transcription factors Gcn4p and Bas1p play important roles in regulating these events.

Intracellular Levels of L-Serine Decrease during Adaptation to Anaerobiosis, and Gcn4p Is Required to Prevent Complete Loss of L-Serine—Increased demand for L-serine during adaptation to anaerobiosis should be reflected in the intracellular concentrations of L-serine, glycine, L-threonine, and L-cysteine, which are metabolically linked to L-serine. Therefore, cells of the wild-type and gcn4 mutant grown aerobically to log phase in SDC medium were transferred to anaerobic conditions, and samples harvested for amino acid analyses at intervals over 6 h.

Intracellular L-serine increased briefly in the wild type, followed by a decrease to 30% of the aerobic level over the 6-h period, and similar changes occurred in the concentrations of glycine and L-cysteine (Fig. 6). Intracellular levels of all of the other amino acids did not show similar decreases, and for L-aspartate and L-asparagine there was a substantial increase in the gcn4 mutant (Fig. 6). In the gcn4 mutant intracellular L-serine was completely depleted by 6 h, indicating that, in the absence of Gcn4p, the cell could not produce sufficient L-serine to meet demand. Glycine also decreased in the gcn4 mutant to ~20% of its level in aerobic cells. L-Threonine increased over the same period, but it was present in the SDC medium used. In SD medium containing L-serine but lacking added L-threonine, levels of threonine also decreased in the wild type and to a greater extent in the gcn4 mutant (data not shown). In S. cerevisiae glycine is mainly derived from L-threonine by the action of threonine aldolase encoded by GLY1 (35). L-Aspartate is the precursor to L-threonine, and its accumulation in the gcn4 mutant indicates that Gcn4p may directly or indirectly regulate the flow from L-aspartate to glycine. During adaptation to anaerobiosis there is therefore a marked alteration of metabolic systems and an increased demand for L-serine. This leads to an increased metabolic demand for glycine to maintain one-carbon metabolism and serves as a precursor for L-serine. Because glycine is mainly synthesized via threonine aldolase (Gly1p) activity, the anaerobic regulation of GLY1 expression was examined.

GLY1 Expression Shows Gcn4p-dependent Induction during Anaerobiosis—The wild-type, gcn4, and bas1 strains were transformed with a GLY1::lacZ reporter, and expression of β-galactosidase determined in each strain after the shift to anaerobic SDC and SDC supplemented with glycine, serine, or formate. In SDC minimal medium, GLY1 expression in the wild type increased 4 h after the shift to anaerobic conditions, and continued to increase up to 24 h (Fig. 7A). This induction under anaerobic conditions was considerably decreased in both gcn4 and bas1 mutant strains, such that at 24 h, GLY1::lacZ expression was more than five times lower in the gcn4 and bas1 strains than in wild type. However, in the presence of added L-serine or formate, expression of GLY1 was low throughout the time course, irrespective of the strain. In contrast, glycine supple-
mentation only repressed expression in the bas1 mutant and decreased GLY1::lacZ expression in the wild type only after 16 h of induction (Fig. 7B).

These data show that GLY1 was strongly induced under anaerobic conditions in the absence of exogenous L-serine. This induction was dependent on both Gcn4p and Bas1p, although neither transcription factor was necessary for basal transcription of GLY1. Because L-threonine accumulated in the gcn4 strain under the same conditions, these data support the hypothesis that cells respond to the reduction in the availability of free L-serine during adaptation to anaerobiosis by increasing the conversion of L-threonine to glycine for use in one-carbon metabolism.

Specific Requirement for L-Serine for Protein Synthesis—What is the basis for such a specific demand for L-serine as cells adapt to anaerobic growth? L-Serine is a precursor not only for protein synthesis and one-carbon metabolism, but also synthesis of cysteine, glutathione, and phospholipids via phosphatidylserine.

An additional requirement for phospholipid synthesis during the switch to anaerobiosis is unlikely, because choline did not rescue the adaptation lag in a gcn4 mutant (Table 3), and labeling with [L-3H]serine did not show major differences between aerobic and anaerobic cells in the extent or distribution of the label entering into lipids.

It is unlikely that there is an additional need for L-cysteine or glutathione under anaerobic conditions, because addition of L-cysteine only partially relieved the adaptation lag. Although the cys3 mutant showed a lag during anaerobiosis, the cys4 mutant did not. This may be due to the accumulation of cystathionine at the expense of L-serine in the cys3 mutant further depleting the cell of L-serine (see Fig. 3). Intracellular glutathione decreased markedly during the switch to anaerobiosis in both the wild-type and gcn4 mutants (supplemental Fig. S1), showing that there was no accumulation of glutathione.

A clue to the destination of the additional L-serine comes from inspection of genes that are up-regulated and/or required for anaerobic growth (5). These include a range of cell wall mannoproteins encoded by TIR1–4 and DAN1–4 (36–38), which belong to a family of very serine-alanine-rich mannoproteins, with ~25–30% of the aminoacyl residues being L-serine (supplementary Table S3). Mannoproteins make up ~40% of the cell wall mass. There are ~20 members of this family of cell wall proteins encoded in the S. cerevisiae genome, some (Cwp1–4p) are synthesized aerobically (37, 39), whereas others, including Tir1–4p, Dan1–4p, and Tip1p (36, 37, 40–42), are up-regulated during anaerobic growth, and strains deleted of TIR1, TIR3, or TIR4 can-
Adaptation to Anaerobiosis in *S. cerevisiae*

**DISCUSSION**

The results show that during adaptation to anaerobiosis in *S. cerevisiae* there is a greatly increased demand for synthesis of L-serine for manufacture of anaerobic cell wall manno-proteins, and this needs to be met not only from glycolysis via the pathway requiring SER1, SER2, and SER3/33, but also from increased synthesis from L-aspartate via L-threonine and glycine requiring GLY1. It has previously been noted that SER1 is essential for anaerobic but not aerobic growth in rich medium (45). One reason why there is a shortfall in L-serine synthesis via glycolysis may come from the demonstration that metabolites from the lower section of the glycolytic pathway decrease quite dramatically with decreasing oxygen in the medium; this includes 3-phosphoglycerate, the substrate of the SER1-dependent pathway (46).

Gcn4p clearly has a major role in cell physiology that has not previously been appreciated and for which the *gcn4* mutant exhibits a very strong phenotype not seen under aerobic conditions. It is required for rapid adaptation to anaerobiosis and probably functions through up-regulation of *SHM2, GLY1*, and other genes associated with synthesis of L-serine and one-carbon metabolism such as the glycine-cleavage reaction. The data also show that in minimal medium under anaerobic conditions the Bas1p transcription factor is essential for growth, and this requirement goes beyond its involvement in the synthesis of purines (47), because the addition of adenine did not alleviate the *bas1* mutant phenotype under anaerobic. This requirement could, however, be met by L-serine; hence, under anaerobiosis Bas1p has a major role in synthesis of L-serine as well as purines.

The threonine aldolase Gly1p is required for rapid adaptation to anaerobic conditions in the absence of L-serine, and GLY1 expression is markedly induced under the same conditions. This induction of GLY1 was dependent on both Bas1p and Gcn4p, and the lack of GLY1 induction in the *bas1* and *gcn4* strains may contribute to their respective anaerobic growth defects. In addition, the lack of anaerobic induction of GLY1 in the *gcn4* strain may explain why it accumulates L-threonine, and both of these phenotypes were relieved by L-serine supplementation. Evidence for regulation of GLY1 by Gcn4p was also observed in transcriptomic analysis (11). However, the effect of Gcn4p and Bas1p on GLY1 expression is likely to be indirect, because the GLY1 promoter region lacks a canonical Gcn4p/Bas1p response element and was not bound by either transcription factor in aerobic genome-wide location analyses (48, 49).

Although *S. cerevisiae* has ~20 cell wall genes, some fungal pathogens have many more very serine-rich proteins that show homology to TIR3 from *S. cerevisiae*. *Candida albicans* has many genes that encode serine-rich proteins that are annotated as cell wall proteins induced by stress or antibiotics, or involved in cell growth (8, 36, 40) there must be a period in which the cell wall composition changes (44), which would impose a substantial requirement for L-serine. This also explains the transient nature of the need for L-serine, because once the adjustment in composition occurs the cell can cope with subsequent continued synthesis of the new cell wall manno-proteins.

To test this hypothesis, cells of the wild-type and *gcn4* mutants were transferred from aerobic to aerobic, and separately to anaerobic conditions in the presence of [L-3H]serine, and its incorporation into various cell fractions was determined. [L-3H]Serine was taken up from the medium to a greater extent by the wild type, and by 6 h most of the label was incorporated into trichloroacetic acid-precipitable material, presumably mainly protein (Fig. 8A). A significantly greater proportion of L-serine was incorporated into the cell wall of the wild type during anaerobic conditions (30% at 5 h; 50% by 24 h) compared with aerobic conditions (20% at 5 h and 30% at 24 h; 2-tailed *t*-test, *p* = 0.002 and 0.05, respectively (Fig. 8B)). In the *gcn4* mutant, in which intracellular L-serine synthesis is limited, the effect was very marked with 75% of the added label being incorporated into cell walls 5 h following the shift to anaerobiosis (cf. 22% aerobically, *p* = 0.0003), with a reduced proportion entering lipid and only very low amounts entering organelle (14,000 × g pellet) or ribosomal (100,000 × g pellet) fractions (Fig. 8C).

To examine changes in cell wall protein composition during the shift to anaerobiosis, cell walls were prepared from aerobic and anaerobic cells of the wild-type and *gcn4* mutants at stationary phase under anaerobic conditions, and their composition was analyzed by mass spectrometry. This led to the identification of Tir1p and Tip1p as components only of the anaerobic cell wall of the wild type and the *gcn4* mutant in the presence of L-serine (Table 4 and supplemental Table S3). In the absence of L-serine, these proteins were not detected in the *gcn4* mutant until it had grown (i.e. at 48 h).
adhesion or pathogenicity. If so, then adaptation of these pathogens to tolerate antibiotics may also depend on increased supply of L-serine. Hence an inhibitor of seryl-tRNA synthetase may have antifungal activity, or in combination increase the efficacy of available antifungal antibiotics. Klis and his colleagues (40) have carried out an elegant investigation of the mannoprotein composition of the aerobic cell wall. This work needs to be extended to follow changes in composition during the transition between aerobic and anaerobic conditions more extensively, because there are substantial changes in the pattern of expression of the mannoprotein genes following a shift to anaerobic conditions (37).

From a systems biology perspective the data show that there are substantial differences between aerobic and anaerobic growth in gene regulation and metabolic networks. The data also indicate that there is a very strong hierarchy in utilization of L-serine for synthesis of various cell components, cell wall protein biosynthesis.

![FIGURE 8. Incorporation of 3H-labeled L-serine in cell fractions of the wild-type strain BY4743 and the gcn4 mutant following a shift to anaerobiosis.](image)

**TABLE 4**

| Protein | Wild type | gcn4 | 0 h | 24 h | 0 h | 24 h (ser) |
|---------|-----------|------|-----|------|-----|-----------|
| Ccw14p  | 15 (6.7)  | 10 (5.8) | 1 (0.7) | 2 (2.2) | 1 (3.3) | 2 (2.0) |
| Cis3p   | 8 (2.6)   | 14 (8.1) | 7 (5.0) | 3 (3.3) | 3 (10.0) | 4 (8.0) |
| Cth1p   | 12 (5.4)  | 8 (4.7)  | 7 (5.0) | 3 (3.3) | 2 (6.7) | 4 (8.0) |
| Cth2p   | 5 (2.2)   | 1 (0.6)  | 2 (1.4) | 1 (1.1) | 0 0 |
| Cwpip   | 16 (7.2)  | 8 (4.7)  | 8 (5.7) | 7 (7.6) | 15 (16.7) | 5 (10.0) |
| Ecm33p  | 9 (4.0)   | 12 (7.0) | 4 (2.8) | 3 (3.3) | 2 (6.7) | 3 (6.0) |
| Gas1p   | 12 (5.4)  | 6 (3.5)  | 4 (2.8) | 4 (4.3) | 1 (3.3) | 2 (4.0) |
| Gas3p   | 9 (4.0)   | 4 (2.3)  | 10 (7.1) | 7 (7.6) | 0 0 |
| Gas5p   | 11 (4.9)  | 7 (4.1)  | 4 (2.8) | 3 (3.3) | 1 (3.3) | 2 (4.0) |
| Gks1p   | 27 (12.1) | 7 (4.1)  | 15 (10.6) | 8 (8.7) | 0 0 |
| Gks2p   | 0 0 |
| Pir1p   | 12 (5.4)  | 24 (14.0) | 17 (12.1) | 12 (13.0) | 0 (7 (14.0) |
| Pir2p   | 0 0 |
| Ppr3p   | 5 (2.2)   | 0 0 |
| Pmt1p   | 9 (4.0)   | 3 (0.2)  | 4 (2.8) | 5 0 |
| Pmt2p   | 9 (4.0)   | 5 (0.3)  | 7 (5.0) | 6 (6.5) | 0 0 (2.0) |
| Pmt4p   | 6 (2.7)   | 3 (0.2)  | 2 (1.4) | 1 (1.1) | 0 0 |
| Ppr3p   | 5 (1.8)   | 3 (0.2)  | 3 (2.1) | 0 0 |
| Ura1p   | 12 (18.8) | 9 (5.2)  | 20 (14.2) | 16 (17.4) | 0 0 |
| Scw4p   | 5 (2.2)   | 0 0 |
| Tip1p   | 1 (0.1)   | 4 (2.3)  | 1 (1.4) | 2 (2.2) | 2 (6.7) | 2 (4.0) |
| Tir1p   | 1 (0.0)   | 20 (11.6) | 1 (0.7) | 0 0 (16.7) | 9 (18.0) |
| Tir2p   | 0 0 |
| Tir3p   | 0 0 |
| Tos1p   | 2 (0.1)   | 0 0 |
| Ygp1p   | 2 (0.1)   | 7 (4.1)  | 3 (2.1) | 4 (4.3) | 3 (10) | 4 (8.0) |

| Total   | 223 172 141 92 30 50 |

Data in parentheses are percentages of each peptide from a particular protein relative to the total number of cell wall peptides identified in each sample.

* gcn4 mutant was grown under anaerobiosis in L-serine-supplemented SDC medium.
Adaptation to Anaerobiosis in S. cerevisiae

takes priority over other proteins, especially under anaerobic conditions, and when L-serine synthesis is depressed there is a very strong bias toward cell wall mannoprotein synthesis at the expense of soluble and ribosomal proteins. One explanation for this probably lies with the very strong serine codon bias for the cell wall mannoprotein genes. All of them (aerobically and anaerobically expressed) have >70% of the codons as UCU or UCC (for which there are 11 tRNA species in S. cerevisiae) with very few as UCA (3 tRNAs), UCG (1 tRNA), AGC or AGU (4 tRNA) (50) (supplemental Fig. S3). This is in line with what is known of codon bias for highly expressed genes and relative abundance of the corresponding tRNAs (50). On the shift of cells to anaerobiosis, when L-serine levels decrease, translation of the relatively highly expressed cell wall genes is favored due to the strong codon bias of the cell wall proteins toward abundant tRNA species. This ensures that serine is preferentially directed to the cell wall and other highly expressed proteins.

Several interesting questions remain. It is not clear why there is a need for remodeling of cell wall mannoproteins during anaerobiosis nor for an increase in the seryl residues in the anaerobic cell wall. Lai et al. (8) have suggested that almost all of the anaerobic seripauarin genes may be up-regulated to modify cell wall porosity, so that the cell wall can accommodate the transport and processing of any specific requirements during the shift to anaerobiosis. Some of this may reflect changes in composition of the cell membrane resulting from the altered supply of ergosterol and unsaturated fatty acid. The increase in serine residues may reflect a need for more extensive mannosylation of cell wall proteins under these conditions, which might therefore be reflected in the morphology of the mannan layer.

Acknowledgments—We thank Geoff Kornfeld for helpful discussion and advice and Prof. G. H. Braus for plasmid constructs.

REFERENCES

1. Rosenfeld, E., and Beauvoit, B. (2003) Yeast 20, 1115–1144
2. Kwast, K. E., Lai, L. C., Menda, N., James, D. T., III, Aref, S., and Burke, P. V. (2002) J. Bacteriol. 184, 250–265
3. Lai, L. C., Kosorukoff, A. L., Burke, P. V., and Kwast, K. E. (2006) Eukaryot. Cell 5, 1468–1489
4. Piper, M. D., Daran-Lapujade, P., Bro, C., Regenberg, B., Knudsen, S., Nielsen, J., and Pronk, T. (2002) J. Biol. Chem. 277, 37001–37008
5. ter Linde, J. J., Liang, H., Daris, R. W., Steensma, H. Y., van Dijken, J. P., and Pronk, J. T. (1999) J. Bacteriol. 181, 7409–7413
6. Kwast, K. E., Burke, P. V., Staahl, B. T., and Poyton, R. O. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 5446–5451
7. Beckhouse, A. G. (2006) The Transcriptional and Physiological Alterations in Brewers Yeast when Shifted from Anaerobic to Aerobic Growth Conditions. PhD thesis, University of New South Wales, Sydney
8. Lai, L. C., Kosorukoff, A. L., Burke, P. V., and Kwast, K. E. (2005) Mol. Cell. Biol. 25, 4075–4091
9. de Groot, M. J. L., Daran-Lapujade, P., van Breukelen, B., Knijnenburg, T. A., de Hulster, E. A. F., Reinders, M. J. T., Pronk, J. T., Albert, Heck, J. R., and Dawes, I. W. (2004) J. Bacteriol. 186, 3735–3740
10. Reiner, S., Micolod, D., Zeling, G., and Schneiter, R. (2006) Mol. Cell. Biol. 26, 17–20
11. Natarajan, K., Meyer, M. R., Jackson, B. M., Slade, D., Roberts, C., Hinnebusch, A. G., and Marton, M. J. (2001) Mol. Cell. Biol. 21, 4347–4368
12. Hinnebusch, A. G. (2005) Annu. Rev. Microbiol. 59, 407–450
13. Pereira, C. M., Sattlegger, E., Jiang, H.-Y., Longo, B. M., Jaqueta, C. B., Hinnebusch, A. G., Wek, R. C., Mello, L. E. A. M., and Castilho, B. A. (2005) J. Biol. Chem. 280, 28316–28323
14. Sood, R., Porter, A. C., Olsen, D., Cavener, D. R., and Wek, C. (2000) Genetics 154, 787–801
15. Cook, R. I. (2000) Am. J. Clin. Nutr. 72, 1419–1420
16. Piper, M. D., Hong, S. P., Sealey, P., and Dawes, I. W. (2002) FEBS Yeast Res. 2, 59–71
17. Piper, M. D., Hinnebusch, A. G., and Marton, M. J. (2001) Mol. Microbiol. 41, 250–265
18. Hermann, H., Hacker, U., Bandlow, W., and Magdon, V. (1992) Gene (Amst.) 119, 137–141
19. Tong, A. H. Y., and Boone, C. (2006) Methods Mol. Biol. 313, 171–192
20. Rose, M., and Botstein, D. (1993) Methods Enzymol. 101, 167–180
21. Klein, C. L., Kleemann, G. R., Lays, H. L., Link, A. J., and Yates, J. R., III (1998) Anal. Biochem. 263, 93–101
22. Schneiter, R., and Daum, G. (2006) Methods Mol. Biol. 313, 75–84
23. Dever, T. E., Feng, L., Wek, R. C., Cigan, A. M., Donahue, T. F., and Hinnebusch, A. G. (1992) Cell 68, 585–596
24. Daignan-Fornier, B., and Fink, G. R. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 6746–6750
25. Monschau, N., Stahnmann, K. P., Sahm, H., McNeil, J. B., and Bognar, A. L. (1997) FEBS Microbiol. Lett. 150, 55–60
26. Abramova, N. E., Cohen, B. D., Setil, O., Kapoor, R., Davies, K. J. A., and Lowry, C. V. (2001) Genetics 157, 1169–1177
27. Abramova, N. E., Sertil, O., Mehta, S., and Lowry, C. V. (2001) J. Bacteriol. 183, 2881–2887
28. Cohen, B. D., Sertil, O., Abramova, N. E., Davies, K. J. A., and Lowry, C. V. (2001) Nucleic Acids Res. 29, 799–808
29. van der Vaart, J. M., Caro, L. H., Chapman, J. W., Klis, F. M., and Verrips, C. T. (1999) J. Bacteriol. 177, 3104–3110
30. Klis, F. M., Mol, P., Helingwerf, K., and Brul, S. (2002) FEBS Microbiol. Rev. 26, 239–259
31. Kitagaki, H., Shimo, H., and Itoh, K. (1997) Eur. J. Biochem. 249, 343–349
32. Kondo, K., and Inouye, M. (1991) J. Biol. Chem. 266, 17535–17544
33. Horie, T., and Isono, K. (2001) Yeast 18, 1493–1503
34. Lipke, P. N., and Ovalle, R. (1998) J. Bacteriol. 180, 3735–3740
35. Reiner, S., Micolod, D., Zeling, G., and Schneiter, R. (2006) Mol. Cell. Biol. 17, 90–103
36. Wiebe, M. G., Rintala, E., Tammelin, A., Simolin, H., Salasjärvi, L., Toivari, M., Kokkonen, J. T., Kiuru, I., Ketola, R. A., Jouhten, P., Huusokoski, A., Maahemo, H., Ruohonhen, L., and Penttilä, M. (2008) FEBS Yeast Res. 8, 140–154
37. Denis, V., and Daignan-Fornier, B. (1998) Mol. Genet. Genom. 259, 246–255
38. Harbison, C. T., Gordon, D. B., Lee, T. I., Rinaldi, N. J., Maclsaac, K. D., Danford, T. W., Hannett, N. M., Tagne, J. B., Reynolds, D. B., Yoo, J., Jennings, E. G., Zeitlinger, J., Pokholok, D. K., Kellis, M., Rolfe, P. A., Takusagawa, K. T., Lander, E. S., Gifford, D. K., Fraenkel, E., and Young, R. A. (2004) Nature 431, 99–104
39. Mieczkowski, P. A., Dominska, M., Buck, M. J., Gerton, J. L., Lieb, J. D., and Pets, D. (2006) Mol. Cell. Biol. 26, 1014–1027
40. Percudani, R., Pavesi, A., and Ortonello, S. (1997) J. Mol. Biol. 268, 322–330