Disruption of the *SHM*2 gene, encoding one of two serine hydroxymethyltransferase isoenzymes, reduces the flux from glycine to serine in *Ashbya gossypii*[1]

Christina SCHLÜPEN*, Maria A. SANTOS†, Ulrike WEBER*, Albert DE GRAAF*‡, José L. REVUELTA† and K.-Peter STAHMANN*§[2]

*Institut für Biotechnologie 1, Forschungszentrum Jülich GmbH, D-52425 Jülich, Germany, †Departamento de Microbiologia y Genetica, Instituto de Microbiologia y Bioquimica, CSIC/Universidad de Salamanca, Campus Miguel de Unamuno, E-37007 Salamanca, Spain, ‡Metabolic Explorer, Technologiezentrum Jülich, Karl-Heinz Beckurts-Str. 13, D-52428 Jülich, Germany, and §Bio-, Chemie- und Verfahrenstechnik, Fachhochschule Lausitz, Großenhainer Str. 57, D-01958 Senftenberg, Germany

Riboflavin overproduction in the ascomycete *Ashbya gossypii* is limited by glycine, a precursor of purine biosynthesis, and therefore an indicator of glycine metabolism. Disruption of the *SHM*2 gene, encoding a serine hydroxymethyltransferase, resulted in a significant increase in riboflavin productivity. Determination of the enzyme's specific activity revealed a reduction from 3 m-units/mg of protein to 0.5 m-unit/mg protein. The remaining activity was due to an isoenzyme encoded by *SHM*1, which is probably mitochondrial. A hypothesis proposed to account for the enhanced riboflavin overproduction of *SHM*2-disrupted mutants was that the flux from glycine to serine was reduced, thus leading to an elevated supply with the riboflavin precursor glycine. Evidence for the correctness of that hypothesis was obtained from ^{13}C-labelling experiments. When 500 μM 99 % [1-^{13}C]threonine was fed, more than 50 % of the label was detected in C-1 of glycine resulting from threonine aldolase activity. More than 30 % labelling determined in C-1 of serine can be explained by serine synthesis via serine hydroxymethyltransferase. Knockout of *SHM*1 had no detectable effect on serine labelling, but disruption of *SHM*2 led to a decrease in serine (2–5 %) and an increase in glycine (59–67 %) labelling, indicating a changed carbon flux.

Key words: amino acid metabolism, C_1 metabolism, fungi, riboflavin overproduction, *Saccharomyces cerevisiae*.

INTRODUCTION

Riboflavin (vitamin B_2) is the precursor of the coenzymes FMN and FAD, which are mainly needed for dehydrogenases, oxidases and mono-oxygenases. Although micro-organisms and plants form riboflavin, humans and domestic animals have to be provided with nutritional sources of riboflavin. Currently, vitamin B_2 is produced on a large scale by both chemical synthesis and biotechnical processes, with the latter gaining more significance [1,2]. One of the most active natural overproducers of riboflavin is the ascomycete *Ashbya gossypii* [3]. For this reason, the filamentous fungus is utilized for industrial riboflavin overproduction. In its metabolism the availability of the educts GTP and ribulose 5-phosphate is of great importance for increasing riboflavin overproduction, since riboflavin formation was found to be enhanced by supplementation of the culture with precursors of the riboflavin molecule, e.g. ribitol [4], purines [5] and glycine [3,5,6]. Supplementation of the medium with glycine, a precursor of purine biosynthesis, increases riboflavin overproduction in *A. gossypii* in the range of 30 % [6] to 650 % [5], depending on the culture conditions. Incorporation of glycine into the riboflavin molecule has already been demonstrated by Plaut [7] by supplementation with [^{14}C]glycine and subsequent analysis of the riboflavin formed. Therefore the metabolism of glycine was of interest previously. Four pathways of glycine biosynthesis are known in organisms (Scheme 1). In addition to threonine aldolase, glyoxylate aminotransferase and the glycine-cleavage system, serine hydroxymethyltransferase (SHMT) is an important enzyme of glycine metabolism. This pyridoxal 5′-phosphate (PLP)-dependent enzyme catalyses the reversible conversion of glycine and 5,10-methylene-tetrahydrofolate into serine and tetrahydrofolate. Serine and glycine have a significant role in metabolism. Besides being used as monomers in protein biosynthesis, they are needed as precursors of different compounds, e.g. phospholipids (serine) and purines (glycine) [8]. In addition, SHMT occupies a central position in one-carbon (C_1) metabolism, because serine serves as a major donor of C_1 units in both prokaryotes and eukaryotes [9]. C_1 units are required for various cellular processes, such as methylation reactions, and purine, thymidylate and methionine biosynthesis. In heterotrophic eukaryotes, the cytosolic and mitochondrial SHMT isoenzymes are expressed from separate nuclear genes [10]. The present study reports the role of SHMT1 and SHMT2 in the metabolism of *A. gossypii*. Since *A. gossypii* is closely related to *Saccharomyces cerevisiae*, we expected from the current metabolic model that overexpression of *SHM*1 should lead to an increased glycine formation, with the consequence of a boosted riboflavin production. However, although the identified genes showed high levels of homology, their roles in the metabolism are clearly different from that of the *S. cerevisiae* model.

EXPERIMENTAL

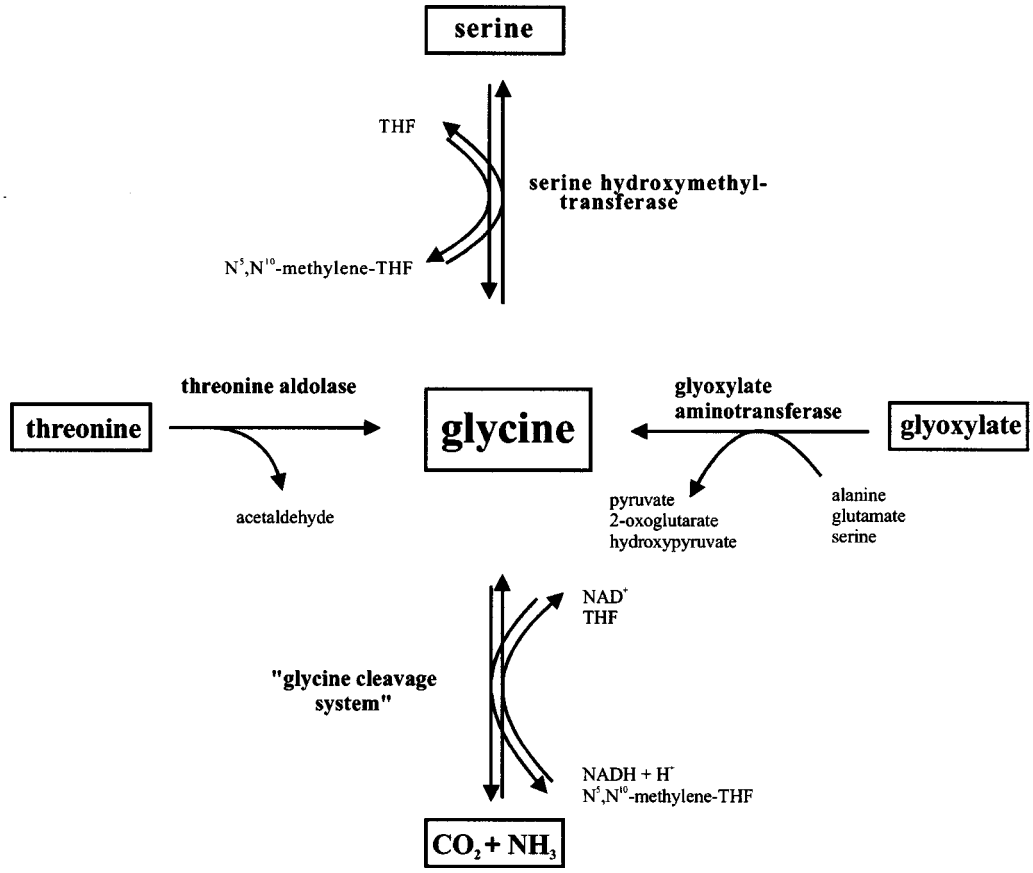
Strains and media

The *A. gossypii* strains *Ag*Δ*SHM*1 and *Ag*Δ*SHM*2 were constructed by creating disruptions at the *SHM*1 or *SHM*2 locus using A.T.C.C. 10895 as the parent. Restriction enzyme-

Abbreviations used: DTT, dithiothreitol; PLP, pyridoxal 5′-phosphate; poly(A)$^+$, polyadenylated; REMI, restriction-enzyme-mediated integration; SHMT, serine hydroxymethyltransferase; SSH, suppression subtractive hybridization; TEF, translation elongation factor.

[1] This paper is dedicated to Professor Dr Hermann Sahm on the occasion of his 60th birthday.

[2] To whom correspondence should be addressed (e-mail P.Stahmann@fz-juelich.de or Stahmann@fh-lausitz.de).



Scheme 1 Enzymic reactions of glycine biosynthesis

Although glycine can be synthesized by the reactions of threonine aldolase, glyoxylate aminotransferase, serine hydroxymethyltransferase and the glycine-cleavage system, only the latter two are reported to catalyse a degradation *in vivo*. THF, tetrahydrofolate.

mediated integration of the *TEF*-promoter-*SHM*1 fragment led to the *SHM*1-overexpressing strain *AgTEF*p*SHM*1. *Escherichia coli* strain DH5α was used as the recipient in all cloning experiments.

A. gossypii strains were maintained on solid rich HA medium consisting of 10 g/l yeast extract, 10 g/l glucose and 20 g/l agar. After 2 days at 30 °C, the plates were stored at 4 °C. On selection, HA medium was supplemented with 400 μg/ml Geneticin or 200 μg/ml hygromycin. For the determination of enzyme activities and riboflavin overproduction, the fungus was grown in liquid HA medium at 30 °C in shaking flasks at 110 rev./min. Growth experiments were performed on solid minimal medium containing 2.5 g/l glucose, 1.5 g/l NH_4Cl, 0.5 g/l asparagine, 0.2 g/l NaCl, 0.4 g/l $MgSO_4 \cdot 7\,H_2O$, 50 mg/l $MnSO_4 \cdot H_2O$, 40 mg/l $CaCl_2 \cdot 2H_2O$, 0.1 g/l *myo*-inositol, 0.25 g/l nicotinic acid amide, 0.2 g/l yeast extract and 20 g/l agar. After sterilization, 2 g/l of KH_2PO_4, pH 6.7, and 0, 1, 10 or 100 mM glycine, serine or threonine was added. For NMR experiments, liquid minimal medium consisting of 1 g/l glucose, 1.5 g/l NH_4Cl, 0.5 g/l asparagine, 0.2 g/l NaCl, 0.4 g/l $MgSO_4 \cdot 7\,H_2O$, 50 mg/l $MnSO_4 \cdot H_2O$, 40 mg/l $CaCl_2 \cdot 2H_2O$, 0.1 g/l *myo*-inositol, 0.25 g/l nicotinic acid amide, 0.05 g/l yeast extract and 0.2 g/l yeast nitrogen base without amino acids and $(NH_4)_2SO_4$ was used. Transformed *E. coli* strains were grown in 2 × TY medium (10 g/l yeast extract, 16 g/l tryptone and 5 g/l NaCl) at 37 °C supplemented with 50 μg/ml ampicillin.

Isolation of *AgSHM*1 and *AgSHM*2

*AgSHM*2 was isolated as one of the positive cDNA clones belonging to a cDNA library constructed by suppression subtractive hybridization (SSH). *A. gossypii* strain A.T.C.C. 10895 spores were inoculated in liquid media consisting of SYG medium (10 g/l soya-bean oil, 10 g/l yeast extract and 6 g/l glycine), and grown on a rotary shaker at 120 rev./min. Samples were taken in the non-production growth phase (after 24 h growth) or riboflavin-production phase (after 72 h growth).

SSH was performed with cells between the non-production growth phase ('tester') and riboflavin production phase ('driver'). For the preparation of polyadenylated [poly(A)$^+$] RNA, total RNA was extracted with RNAzol (Biotecx Laboratories, Houston, TX, U.S.A.) reagent and purified with oligo-dT cellulose columns (Amersham). Further steps were performed according to the PCR-Select™ cDNA subtraction kit (Clontech). For the first-strand cDNA synthesis, 2 μg of the tester- and driver-poly(A)$^+$ RNA was incubated with oligo-dT primer for 2 min at 70 °C, and rapidly cooled on ice. The reverse-transcription reaction was carried out in RT buffer [50 mM Tris/HCl (pH 8.3)/6 mM $MgCl_2$/75 mM KCl] with 1 mM dNTPs and 200 units of Moloney murine leukaemia virus ('MMLV') reverse transcriptase for 90 min at 42 °C. After this reaction, second-strand cDNA synthesis followed immediately by incubating the first-strand cDNA for 2 h at 16 °C in 100 mM KCl, 10 mM

ammonium sulphate, 5 mM $MgCl_2$, 15 mM NAD, 20 mM Tris/HCl, pH 7.5, 0.05 mg/ml BSA containing 1 mM dNTPs, 0.3 units/μl DNA polymerase I, 0.01 unit/μl RNase H and 0.06 unit/μl *E. coli* DNA ligase. The double-stranded cDNA was blunted by T4 DNA polymerase for 30 min at 16 °C. The reaction was stopped by the addition of EDTA/glycogen, followed by the precipitation of the cDNA.

The adapter ligation was performed only with the reverse-transcribed and digested mRNA of the tester cDNA. One-half of the tester cDNA was ligated with 2 μM adapter-1, the other half with 2 μM adapter-2 in the ligation mixture [50 mM Tris/HCl (pH 7.8)/10 mM $MgCl_2$/1 mM dithiothreitol (DTT)/1 mM ATP/5 % poly(ethylene glycol)/0.5 unit of T4 DNA ligase] at 16 °C overnight. The reaction was stopped by EDTA/glycogen and the enzyme was inactivated by heating the samples at 72 °C for 5 min. For the first hybridization, an excess of driver cDNA was added to each tester cDNA (ligated with adapter-1 or -2) in separate samples. After denaturation at 98 °C for 2 min, the first hybridization was performed in a hybridization buffer at 68 °C for 8 h. For the second hybridization, the two samples of the first hybridization were combined without denaturation at 68 °C overnight.

A primary PCR was used to selectively amplify the differentially expressed sequences, and performed with 1 μl of the diluted subtracted cDNA in a 25 μl volume containing 400 nM of each primer, 0.2 mM dNTPs and 0.5 μl of Advantage Klen*Taq* polymerase mix (Clontech). PCR was performed in a thermal cycler (PTC-100; MJ Research, Watertown, MA, U.S.A.) with the following parameters: 75 °C for 5 min, 30 cycles at 94 °C for 30 s, 66 °C for 30 s and 72 °C for 90 s. Then, 1 μl of the amplified product was used as a template in the secondary PCR for 30 cycles with nested adapter-specific primers under the following conditions: 94 °C for 5 min, 30 cycles at 94 °C for 30 s, 68 °C for 30 s and 72 °C for 90 s.

The subtracted cDNA fragments were then inserted into the pGEM-T cloning vector (Invitrogen) and transformed into DH5α cells. To confirm the differential expression of the selected clones, Northern blots and semi-quantitative reverse transcriptase-PCR analysis were performed by standard methods [11]. DNA sequencing of randomly selected clones was performed by the dideoxy chain reaction [12] in the ABI Prism 310 Genetic Analyser sequencing system (PerkinElmer) and nucleic acid homology searches were carried out using the BLAST program [13].

One of the positive clones (pJR2400) showing a level of expression in the growth phase higher than that in the production phase contained an insert capable of encoding a polypeptide homologous with SHMTs from various organisms, and was used as a radiolabelled probe to screen an *Ashbya* genomic library constructed in the cosmid vector SuperCos1 (Stratagene), available on request from M. A. Santos or J. L. Revuelta. Cosmid DNA of one of the positive clones (pJR2128) was cut with *Hin*dIII and again probed using the radiolabelled cDNA fragment. To obtain smaller fragments of genomic DNA for sequencing, positive DNA fragments were subcloned into the plasmid vector Bluescript (Stratagene).

*AgSHM*1 was identified by screening the cosmid library under low-stringency conditions using as a probe a 921 bp *Bgl*II fragment that corresponded to the coding sequence of *AgSHM*2. A weakly hybridizing clone, pJR2145, which showed a restriction pattern different from that of the *AgSHM*2 cosmid pJR2128, was isolated and the restriction fragments that hybridized with the probe were subcloned and sequenced.

Nucleotide sequences of the *AgSHM*1 and *AgSHM*2 genes are available under the EMBL Data Bank accession numbers AJ438778 and AJ438779 respectively.

Replacement mutagenesis

For *SHM*1 disruption, a 769 bp *Xho*I–*Sal*I fragment encompassing part of *AgSHM*1 open reading frame was replaced with a 2.1 kb G418r cassette. *A. gossypii* A.T.C.C. 10895 was transformed with the 2.7 kb *Bam*HI–*Kpn*I fragment of the plasmid pJR1550 *SHM*1Δ769::G418r. The resulting strain was designated *Ag*Δ*SHM*1. For disruption of *SHM*2, a 1.3 kb *Sal*I–*Eco*RV fragment of the plasmid pJR2417 was deleted, and a 1.6 kb *Bam*HI–*Hin*dIII fragment containing the hygromycin marker was inserted. A 2.1 kb linear fragment containing the *SHM*2Δ1300::hygromycin marker was obtained by digestion of the plasmid pJR2427 with *Sph*I and used to transform *A. gossypii*. The resulting strain was designated *Ag*Δ*SHM*2.

Chromosomal integration

A 4.86 kb fragment consisting of the Geneticin-resistance marker under the control of the promoter of the translation elongation factor (*TEF*) gene and terminator, and the *SHM*1 gene controlled by *TEF* promoter and *RIB*5 terminator, was obtained by digestion of the plasmid pAGTGT*SHM*1 with *Spe*I. Restriction-enzyme-mediated integration (REMI) into *A. gossypii* wild-type was performed by adding 50 extra units of *Spe*I to the electroporation mixture. The Geneticin-resistant and *SHM*1-overexpressing transformant was designated *AgTEF*p*SHM*1.

DNA isolation, DNA modification, Southern blot analysis, PCR amplification and transformation

Restriction endonucleases, T4 DNA ligase, Klenow DNA polymerase and other enzymes were purchased from Roche Diagnostics GmbH. Restriction enzyme digestions and ligation of DNA fragments were carried out according to the manufacturer's instructions. Plasmids were prepared by the alkaline lysis method, as described by Sambrook and Russell [11]. Isolation of genomic DNA of *A. gossypii* was performed using the DNeasy Plant Maxi Kit (Qiagen).

DNA–DNA hybridization was carried out as described by Southern [14] with the appropriate probes at 65 °C. Genomic DNA (30 μg) was digested and isolated by gel electrophoresis. The DNA was transferred to a nylon membrane (Hybond™-N+; Amersham Biosciences, Freiburg, Germany). As a template for the probe to prove the *SHM*2 disruption, an *Eco*RI–*Eco*RV fragment of the *SHM*2 gene was isolated from the agarose gel using the QIAquick Gel Extraction Kit (Qiagen) and labelled by the random hexanucleotide procedure using Klenow fragment of DNA polymerase I. The *SHM*1-overexpressing transformants were also investigated by Southern blot analysis. A 0.75 kb *Sal*I–*Mun*I fragment of the *SHM*1 gene was applied as a probe template. For the detection of hybridization signals, the DIG DNA Detection Kit (Roche) was used.

For the amplification of gene fragments from isolated DNA, PCR was performed with the Expand High Fidelity PCR System (Roche). The 50 μl standard sample consisted of 1–5 ng of template DNA, one-tenth vol. of PCR buffer (10×), 1/50 vol. of dNTP mixture (2 mM dATP, dTTP, dCTP and dGTP), 1 μM primer and 1.5 units of Expand High Fidelity PCR polymerase. The amplification was carried out in PTC-100 Programmable Thermal Controller (MJ Research). To verify the *SHM*1 disruption, the primers *SHM*-UP (5′-AAGCCGCACAGTGTTGCACGAAGGCGTATG-3′) and *SHM*-DOWN (5′-TGTTTCTGGCCCTGTAACCAATAATTCATG-3′) were used.

Transformation of *A. gossypii* mycelium was done by electroporation. The cells were cultivated overnight in rich medium. The mycelium was harvested, resuspended in 50 mM potassium

phosphate, pH 7.5 containing 25 mM DTT and incubated at 30 °C for 30 min. After filtration, the non-diffusible material was resuspended in ice-cold STM [270 mM saccharose/10 mM Tris/HCl (pH 7.5)/1 mM $MgCl_2$]. Electroporation of 150 μl of cell suspension with 1–5 μg of DNA was carried out at 1500 V/cm and 25 μF (Gene Pulser; Bio-Rad Laboratories, Munich, Germany). The transformed cells were plated on rich medium and incubated at 30 °C for 6 h. Subsequently, the cells were overlaid with 6 ml of top agar [50 mg of agarose type VII (Sigma) in rich medium containing Geneticin or hygromycin]. Transformants were obtained after more than 5 days.

Spore isolation

For the isolation of ascospores, *A. gossypii* was cultivated for 4–6 days on solid rich medium. The resulting mycelium was resuspended in a solution of Glucanex™ [Novo Ferment AG, Basel, Switzerland; 10 mg/ml in 0.9 % (w/v) NaCl] and incubated at 30 °C for 1 h in order to release the spores from the asci. Subsequently, the suspension was centrifuged at 3000 rev./min (900 ***g***) for 5 min. The resulting pellet was washed three times with 0.9 % NaCl, resuspended in a small volume of 0.9 % (w/v) NaCl, and finally the same volume of high-viscosity paraffin was added. After vigorous shaking and subsequent phase separation, the hydrophobic spores of *A. gossypii* were found in the upper paraffin layer, while cell debris remained in the aqueous phase. The spore suspension could be stored for several months at 4 °C.

Determination of total riboflavin and mycelial dry weight

In order to determine total riboflavin, cell walls were degraded by the addition of 2.5 mg of Glucanex™ (Novo Ferment AG) to 0.5 ml of a sample taken from liquid cultures. After incubation at 30 °C for 1 h, 450 μl of distilled water was added. The resulting homogenate was centrifuged for 5 min at 13000 rev./min (15500 ***g***), filtered (0.45 μm pore size; Millipore, Eschborn, Germany) and analysed for riboflavin by HPLC as described by Schmidt et al. [15]. For the determination of mycelial dry weight, samples were taken from liquid cultures and filtered through paper filters. The mycelium was dried overnight at 110 °C and weighed.

Cell extraction

A. gossypii mycelium was harvested by filtration, rinsed with distilled water and resuspended in 50 mM Hepes/NaOH buffer, pH 7.0, containing 1 mM DTT and 20 μM PLP at a ratio of 2–5 ml/g wet weight. The cells were disrupted in a French press (Aminco, Silver Spring, MD, U.S.A.) at 138000 kPa, and the resulting homogenate was centrifuged at 20000 ***g*** for 20 min. The supernatant was used in enzyme assays, and is subsequently referred to as the crude extract. All procedures were carried out at 4 °C.

Enzyme assay

SHMT activity was assayed according to a modification of the method described by Geller and Kotb [16]. The incorporation of radioactivity from L-[3-^{14}C]serine into 5,10-methylene-tetrahydrofolate was measured. Enzyme assays were performed in 50 mM Tris/HCl, pH 8.0, containing 0.2 mM serine, 0.024 mM L-[3-^{14}C]serine (2 GBq/mmol; Amersham Biosciences), 2 mM tetrahydrofolic acid (6*R*,6*S* racemic mixture, T 3125; Sigma–Aldrich Fine Chemicals, Taufkirchen, Germany), 0.25 mM PLP, 2.5 mM EDTA, pH 8.0, 3 mM DTT and crude extract. The labelled 5,10-methylene-tetrahydrofolate was separated from serine by streaking a 25 μl aliquot of the assay mixture on to small DEAE-cellulose filter circles (Whatman DE 81) and subsequent washing of the filters in distilled water. Protein concentrations were determined spectrophotometrically by the method of Bradford [17] at 595 nm with the Serva Blue G dye binding reagent. BSA was used as a standard.

^{13}C-labelling experiments

A. gossypii was cultured on media containing 500 μM [1-^{13}C]serine or 500 μM [1-^{13}C]threonine. Each set of experiments consisted of nine shaker flasks (500 ml) per strain containing 100 ml of medium. The inoculum was grown in a minimal medium containing 0.2 g/l yeast extract instead of a concentration of 0.05 g/l. During growth, the uptake of amino acids was monitored by HPLC. After 72 h, the contents of all nine flasks were pooled together, the mycelium was harvested by filtration and then freeze-dried. Acidic hydrolysis of 200 mg of biomass by 6 M HCl for 12 h at 105 °C revealed a sufficient concentration of amino acids from the cellular protein content to purify them by FPLC. Finally, ^{13}C enrichments were determined by spin-echo difference spectroscopy [18] using a Bruker AMX 400-WB spectrometer system at 100.61 MHz.

RESULTS

Sequence analysis of the *A. gossypii SHM*1 and *SHM*2 genes

Translation of the *AgSHM*1 and *AgSHM*2 open reading frames revealed that they code for proteins of 497 amino acids and 469 amino acids respectively. Cytosolic and mitochondrial SHMTs have been identified from *S. cerevisiae* [19] and *Candida albicans* [20]. The amino acid sequences for the published SHMT isoforms aligned to *A. gossypii* SHMT1 and SHMT2 are shown in Figure 1. Highest identities were found for *Ag*SHMT2, sharing 85 % congruent amino acids with the cytosolic SHMT2 from *S. cerevisiae* and 80 % with the cytosolic enzyme from *C. albicans*. On the other hand, *Ag*SHMT1 was found to fit best with the mitochondrial isoenzymes (68 % identical amino acids with *Sc*SHMT1; 67 % with *Ca*SHMT1). Homology was confirmed by the identification of a tetrapeptide sequence (Thr-Thr-His-Lys) that is conserved in all eukaryotic and prokaryotic SHMT proteins in the SWISS-PROT protein sequence database. This consensus sequence contains a lysine residue at position 248 in *Ag*SHMT2 and at position 272 in *Ag*SHMT1, which is equivalent to the lysine residue at position 257 in the human SHMTs and at position 229 in *E. coli* SHMT. Biochemical investigations with enzymes purified after site-directed mutagenesis have shown that this lysine is involved in PLP binding to SHMT [21]. Genetic studies and crystal-structure analysis of the human SHMT

Figure 1 Alignment of SHMT proteins from related hemiascomycetes

The alignment was obtained by CLUSTAL-W analysis of the deduced protein sequences from the *SHM* genes from *A. gossypii* (*Ag*) [AJ438778 (SHMT1) and AJ438779 (SHMT2)], *S. cerevisiae* (*Sc*) [P37292 (SHMT1) and P37291 (SHMT2)] and *C. albicans* (*Ca*) [O13425 (SHMT1) and O13426 (SHMT2)]. Identical amino acids are shown in reversed-out lettering on a black background; conserved substitutions are on a grey background. The tetrapeptide sequence that is conserved in all eukaryotic and prokaryotic SHMT proteins is shown double-underlined. Residues implicated in the enzyme catalytic site are enclosed in boxes. The position of the active-site lysine residue, which binds the pyridoxal cofactor, is indicated by an asterisk. The underlined amino acid residues depict the putative mitochondrial-targeting sequence.

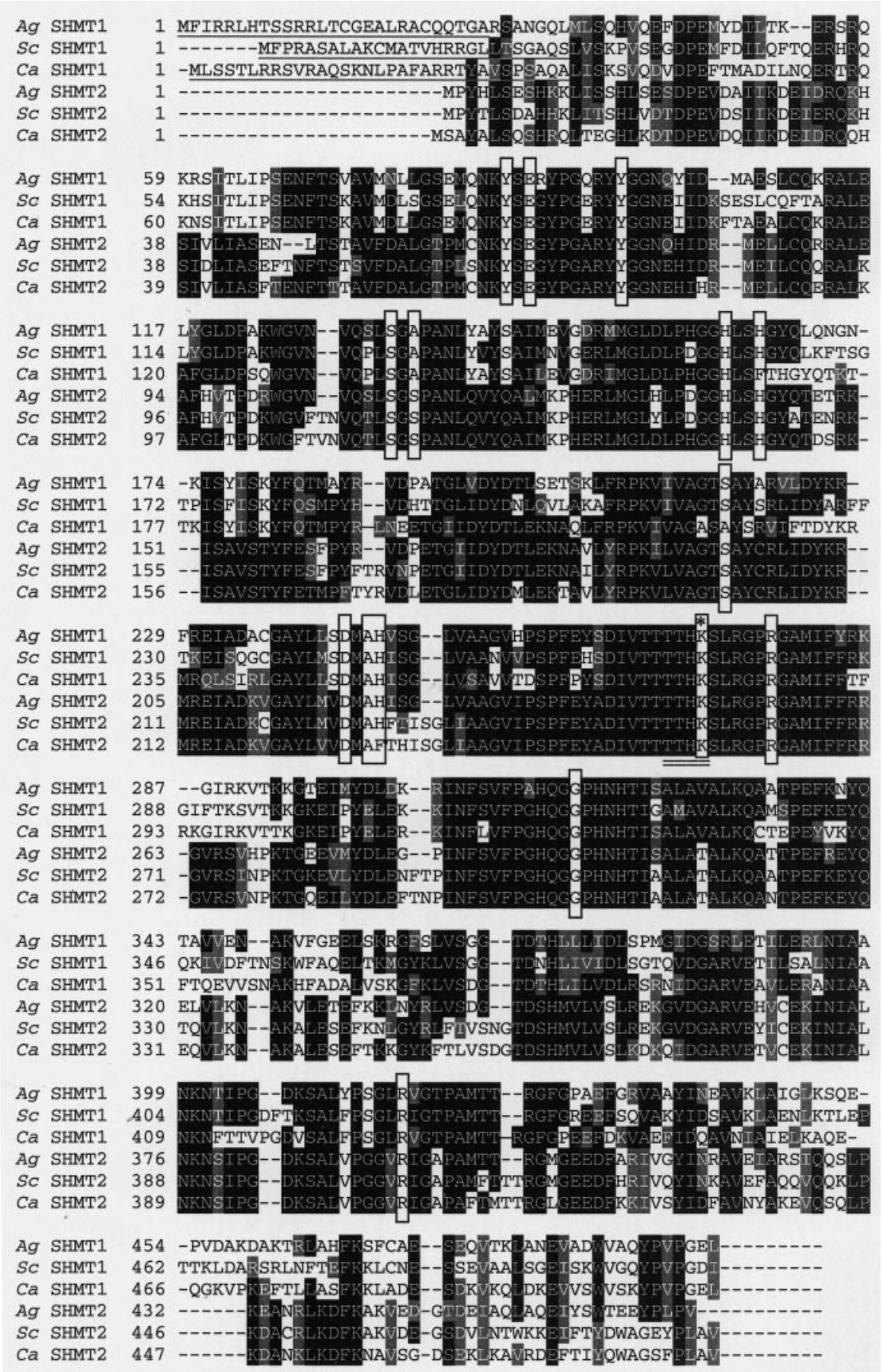


Figure 1 For legend, see facing page

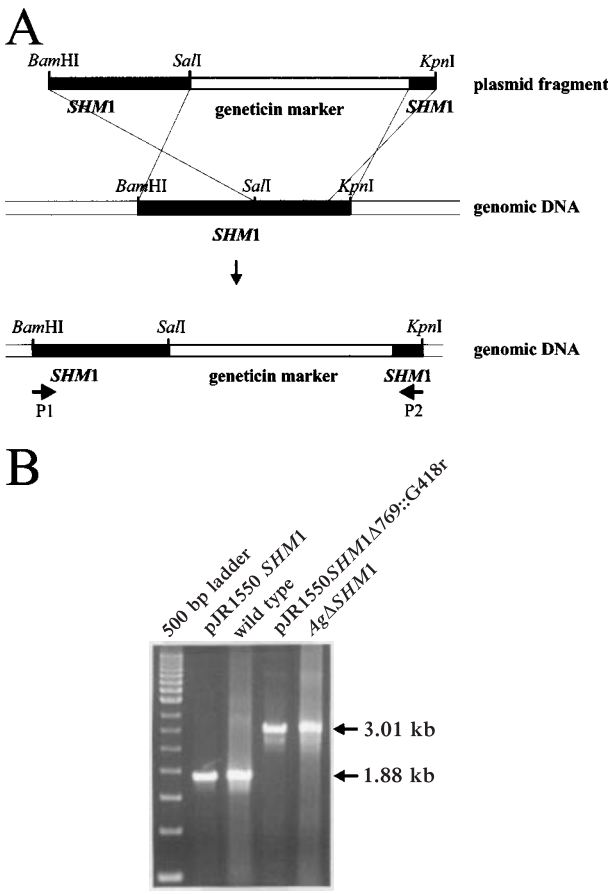


Figure 2 Disruption of *AgSHM1*

(**A**) For *SHM*1 disruption, *A. gossypii* wild-type (A.T.C.C. 10895) was transformed with the *SHM*1Δ769::G418r fragment (2.7 kb), which was obtained by *Bam*HI–*Kpn*I digestion of plasmid pJR1550 *SHM*1Δ769::G418r (results not shown). By a double crossing-over event, the *SHM*1 gene was replaced with the disruption cassette. Transformants were analysed by PCR. P1 and P2 represent the used primer (the Figure is not to scale). (**B**) Agarose-gel electrophoresis of PCR products using, as a template, pJR1550 *SHM*1, wild-type DNA, pJR1550 *SHM*1Δ769::G418r and *AgΔSHM*1 DNA.

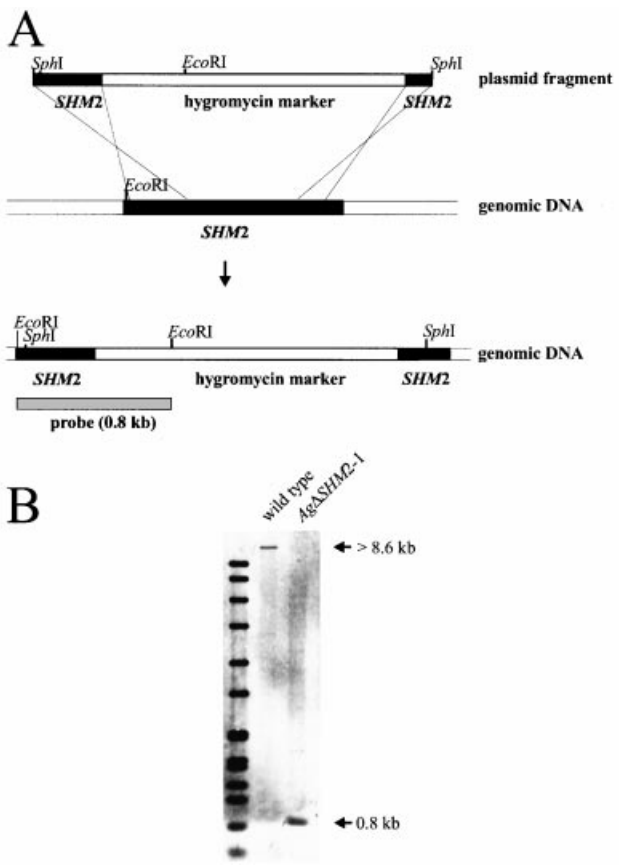


Figure 3 Disruption of *AgSHM2*

(**A**) *A. gossypii* was transformed with an *Sph*I fragment of plasmid pJR2427 (results not shown) consisting of *SHM*2Δ1300::hygromycin (Figure is not to scale). By homologous replacement mutagenesis, the *SHM*2 gene was replaced by the disruption cassette. Hygromycin-resistant strains were analysed by DNA: DNA hybridization. Therefore a probe representing the 0.8 kb *Eco*RI fragment of the *SHM*2 disruption cassette was used. (**B**) Southern blot analysis: genomic DNA of wild-type and *AgΔSHM2*-1 were digested with *Eco*RI. The locus of the next *Eco*RI site vicinal to *SHM*2 in the *A. gossypii* genome is unknown.

proteins have identified 15 further amino acid residues involved in catalysis. Of these, 14 residues also occurred in the investigated fungal SHMTs (Figure 1). Supporting these alignment results, the PSORT II Prediction program proposed the first 27 amino acids of *Ag*SHMT1 as a putative mitochondrial targeting sequence (Figure 1).

Effect of *SHM* disruption on SHMT specific activity

To study the function of the *SHM* gene products, the chromosomal *SHM*1 and *SHM*2 genes were inactivated by gene disruption. For the *SHM*1 disruption experiment, the *SHM*1Δ769::G418 fragment was liberated from the plasmid by *Bam*HI–*Kpn*I digestion, and *A. gossypii* wild-type was transformed by electroporation, thereby inducing DNA integration by homologous recombination. PCR confirmed the disruption of the *SHM*1 gene (Figure 2). Similarly, for the *SHM*2 disruption experiment the linear *Sph*I fragment containing the hygromycin-resistance marker flanked by *SHM*2 DNA was used to transform the *A. gossypii* wild-type strain to hygromycin resistance due to homologous replacement mutagenesis. Southern analysis confirmed the *SHM*2 disruption (Figure 3). To investigate whether the disruption of *SHM*1 or *SHM*2 led to a detectable loss in enzyme activity, a sensitive radioactive SHMT assay was performed. In *AgΔSHM*1, no difference in SHMT specific activity in comparison with the wild-type was detectable. However, the disruption of *SHM*2 caused an 85% decrease of activity compared with the wild-type (Table 1). This led to the conclusion that *SHM*2 encodes a functional SHMT and that SHMT2 contributes the major part of *in vitro* activity. The remaining activity detected in *AgΔSHM*2 supposed the existence of at least one isoenzyme. This isoenzyme could be encoded by *SHM*1. Because SHMT1 contributes to SHMT specific activity to a lesser extent, i.e. in the magnitude of the scattering, a difference in activity compared with the wild-type was not detectable.

REMI of *TEF*-promoter-*SHM*1 and effect on specific activity

To study the functionality of the *SHM*1 gene, *SHM*1-overexpressing strains were constructed. *Spe*I digestion of plasmid pAG TGT*SHM*1 (results not shown) released a fragment con-

Table 1 SHMT specific activity and riboflavin overproduction of strains disrupted in *SHM*

A. gossypii wild-type and *SHM* mutant strains were cultivated on rich medium. After 72 h, riboflavin production was determined and a sensitive radioactive assay was performed to measure SHMT specific radioactivity in crude extracts, as described in the Experimental section. To obtain total enzyme activity, the detected activities in boiled crude extracts were subtracted. Triplicate investigations were carried out per strain. Two independent strains were investigated in the case of *SHM*2 replacement. Means ± S.D. are shown.

Strain	SHMT specific radioactivity (m-unit/mg of protein)	Productivity (mg of riboflavin/g biomass)
Wild-type	2.8 ± 0.1	0.9 ± 0.1
*Ag*Δ*SHM*1	2.6 ± 0.2	1.1 ± 0.2
*Ag*Δ*SHM*2-1/2	0.5 ± 0.0	9.6 ± 1.0

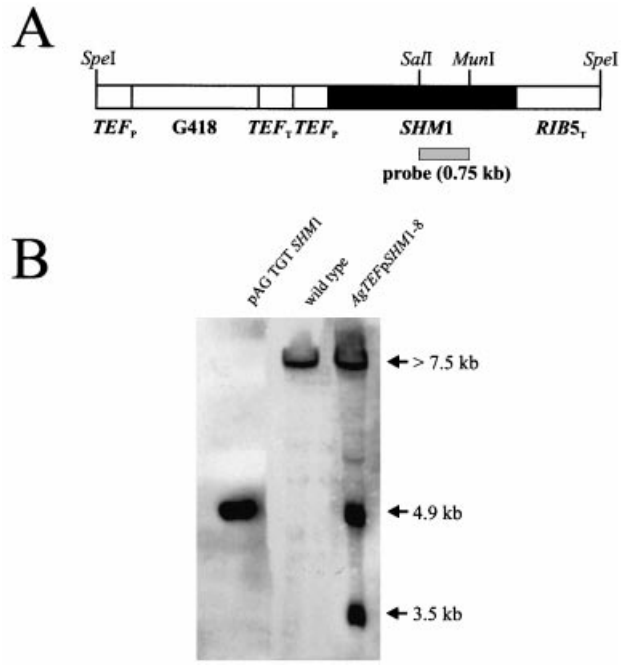


Figure 4 REMI of *TEF*-promoter-*SHM*1

(**A**) *Spe*I fragment of plasmid pAG TGT *SHM*1 utilized for REMI (not to scale). The 4.86 kb fragment contains the Geneticin (G418)-resistant marker under the control of *TEF* promoter (TEF_P) and terminator (TEF_T) and the *SHM*1 gene under the control of TEF_P and *RIB*5 terminator ($RIB5_T$). For Southern blot analysis a 0.75 kb *Sal*I–*Mun*I fragment was used as a template for the random hexanucleotide method. (**B**) Southern blot analysis of plasmid pAG TGT *SHM*1 and genomic DNAs of wild-type and the REMI transformant *AgTEF*p*SHM*1-8 after *Spe*I digestion. Loci of *Spe*I sites in the vicinity of *SHM*1 are unknown.

taining the *SHM*1 gene under the control of the strong *TEF* promoter (Figure 4). Transformants were investigated that resulted from REMI events by Southern analysis of a genomic DNA *Spe*I digestion. As the probe template, a 0.75 kb *Sal*I–*Mun*I fragment of *SHM*1 was used. Insertion of the transformed *Spe*I fragment should result in a single 4.86 kb signal if the fragment was inserted into a genomic *Spe*I site. The result of the Southern blot analysis is depicted in Figure 4. *Spe*I restriction of the plasmid led to the expected signal. The signal of the genomic digestion of wild-type represented the natural locus of the *SHM*1 gene. Genotypic characterization of 30 REMI transformants by

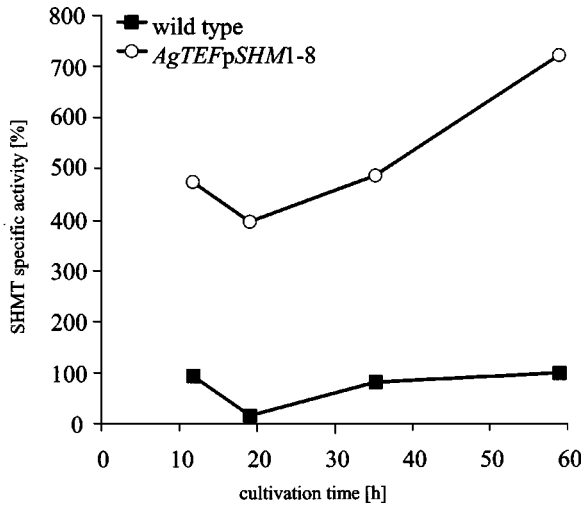


Figure 5 SHMT specific activity of *AgTEF*p*SHM*1

Comparison of SHMT specific activity in wild-type (■) and *AgTEF*p*SHM*1-8 (○) cultivated for 60 h in rich medium. Each datum represents the mean of at least three independent measurements. Standard deviations (not shown) were less than 30%.

PCR and Southern blot hybridization revealed single and multiple, as well as complete and incomplete, integration (results not shown). *AgTEF*p*SHM*1-8 was one of three 'true' REMIs, which means that probably an insertion took place in a genomic *Spe*I site, because it was possible to cut the *Spe*I fragment out of the isolated genomic DNA (Figure 4B). Hence this strain was used to investigate the effect on SHMT specific activity resulting from integration of an additional *SHM*1 gene under the control of the *TEF* promoter. At various points in time over a 60 h period of cultivation, the SHMT specific activity of *AgTEF*p*SHM*1-8 was determined in comparison with the wild-type. Figure 5 shows a 6-fold increase in activity in *AgTEF*p*SHM*1-8, suggesting that the endogenous promoter of *SHM*1 is much weaker. These results show that the *SHM*1 gene codes for a functional SHMT isoenzyme, designated SHMT1. Compared with SHMT2, SHMT1 contributes less to the SHMT specific activity *in vitro*.

Effect of *SHM* disruption on riboflavin overproduction

The transformants *Ag*Δ*SHM*1 and *Ag*Δ*SHM*2 were used to study the effect of the *SHM* disruption on riboflavin overproduction. Whereas *Ag*Δ*SHM*1 produced the same amount of riboflavin as the wild-type, the production of *Ag*Δ*SHM*2-1 increased 10-fold (Table 1). These data raised the question of whether this alteration of riboflavin overproduction correlates with a change in the carbon flux to glycine. In order to investigate whether more glycine was available for the riboflavin biosynthesis in *Ag*Δ*SHM*2, ^{13}C-labelling experiments were performed.

Role of SHMT1 and SHMT2 *in vivo*

Since a conversion of glycine into serine competes with the glycine amide ribonucleotide synthetase reaction, which limits the precursor supply for riboflavin overproduction, inactivation of a SHMT could explain the enhancement found in the productivity of *Ag*Δ*SHM*2. Labelling experiments were performed to study the function *in vivo* of SHMT1 and SHMT2.

In a first and second set of experiments, 500 μM [1-^{13}C]serine was fed to analyse the *in vivo* conversion into glycine (Table 2).

Table 2 Metabolism of [1-^{13}C]serine by *SHM*1 mutants

Percentages of ^{13}C enrichments in C-1 of serine, glycine and threonine obtained from cell protein after growth on minimal medium. Supplementation of wild-type, *Ag*Δ*SHM*1 and *AgTEF*p*SHM*1-8 with [1-^{13}C]serine was performed in two independent experiments, depicted as first and second set. The detected labelling patterns in serine were set as 100% (shown in parentheses).

	^{13}C-labelling of C-1 (%)					
	Serine		Glycine		Threonine	
Strain	First set	Second set	First set	Second set	First set	Second set
Wild-type	64.6 (100)	60.0 (100)	46.5 (71)	53.3 (89)	1.1 (2)	1.1 (2)
Ag Δ *SHM*1	55.1 (100)	60.5 (100)	36.3 (65)	45.2 (75)	1.1 (2)	1.2 (2)
AgTEF$_{P}$*SHM*1-8	61.9 (100)	56.9 (100)	47.6 (76)	46.3 (81)	1.3 (2)	1.0 (2)

Table 3 Metabolism of [1-^{13}C]serine by *SHM*2 mutants

Percentages of ^{13}C enrichments in C-1 of serine, glycine and threonine obtained from cell protein after growth on minimal medium. Supplementation of wild-type and two independent *SHM*2 mutants, *Ag*Δ*SHM*2-1 and *Ag*Δ*SHM*2-2, with [1-^{13}C]serine. The detected labelling patterns in serine were set as 100% (shown in parentheses).

	^{13}C-labelling of C-1 (%)					
	Serine		Glycine		Threonine	
Strain	Third set	Fourth set	Third set	Fourth set	Third set	Fourth set
Wild-type	69.0 (100)	73.7 (100)	56.9 (82)	61.6 (83)	0.8 (1)	2.2 (3)
*Ag*Δ*SHM*2-1	63.1 (100)		29.9 (47)		0.9 (1)	
*Ag*Δ*SHM*2-2		71.0 (100)		35.7 (50)		0.5 (1)

Table 5 Effect of glycine, serine and threonine supplementation on growth of wild-type, *Ag*Δ*SHM*1 and *Ag*Δ*SHM*2

The data represent the growth rate, determined as increase in radius of a colony, in mm/day. Measured was the increase from day 3 to day 5 of incubation at 30 °C. The supplementation of minimal medium (containing glycine $< 45\ \mu$M, serine $< 55\ \mu$M or threonine $< 55\ \mu$M) was with 0, 1, 10 or 100 mM glycine, serine or threonine. Data are means for two experiments. S.D. (not shown) was $< \pm 5$%.

	Growth rates (mm/day)		
Amino acid supplemented (mM)	Wild-type	*Ag*Δ*SHM*1	*Ag*Δ*SHM*2-1
Glycine (0)	6.4	6.1	2.3
Glycine (1)	6.3	6.0	2.3
Glycine (10)	6.3	3.5	2.8
Glycine (100)	4.2	1.0	3.4
Serine (0)	5.6	5.5	2.5
Serine (1)	5.5	5.7	3.0
Serine (10)	1.8	0.5	1.8
Serine (100)	0.0	0.0	0.0
Threonine (0)	5.7	5.8	2.6
Threonine (1)	5.1	5.6	3.4
Threonine (10)	2.7	0.7	0.7
Threonine (100)	1.3	0.5	0.8

The labelling in serine, ranging from 55.1% to 64.6%, indicated a *de novo* synthesis of serine, because initially 99% [1-^{13}C]serine was fed. A dilution by an uptake of unlabelled serine is negligible, because its concentration in the cultivation medium was found to be below 10 μM. Since 89% and 71% of the labelling identified in serine was determined in the glycine of the wild-type respectively, we concluded that SHMT must be the major, but not the only, reaction supplying the glycine pool. No labelling was found in C-1 of threonine, indicating that threonine aldolase is not reversible. No significant change was detectable when *Ag*Δ*SHM*1 or *AgTEF*p*SHM*1 was used for the labelling experiment. We concluded that SHMT1 contributed much less to the serine-to-glycine flux than SHMT2, or the latter was able to compensate for the inactivated enzyme. To show with statistical significance that disruption of *SHM*1 leads to a reduced labelling in glycine, which appears to hold true when one looks at each set of experiments separately, seems impossible concerning the scattering of the data. In a third and fourth set of experiments, *Ag*Δ*SHM*2 strains were investigated (Table 3). Because of the striking enhancement in riboflavin overproduction, a second, completely independent mutant (*Ag*Δ*SHM*2-2) was constructed and investigated. A significant decrease in [1-^{13}C]glycine labelling was detectable. Whereas the wild-type showed 82% and 83% C-1 labelling in glycine, the mutants reached only 47% and 50%, indicating that the remaining SHMT cannot compensate for the inactivation of SHMT2. To investigate the flux from glycine to serine, three further sets (fifth to seventh sets) of labelling experiments were performed, now feeding 500 μM [1-^{13}C]threonine (Table 4). In contrast with the [1-^{13}C]serine experiments, labelling in the fed amino acid was significantly higher (up to 98.4%), indicating a down-regulation of threonine biosynthesis caused by the supplementation. In the wild-type, 52–57% labelling was detected in glycine. Interestingly, 31–35% of the C-1 carbon in serine was ^{13}C-labelled. That this is due to *in vivo* catalysis of SHMT2 is convincingly shown by a reduced labelling of the serine isolated from the two *Ag*Δ*SHM*2 strains.

Table 4 Metabolism of [1-^{13}C]threonine by *SHM*1 and *SHM*2 mutants

Percentages of ^{13}C enrichments in C-1 of serine, glycine and threonine obtained from cell protein after growth on minimal medium. Supplementation of wild-type, *Ag*Δ*SHM*1, *Ag*Δ*SHM*2-1 and *Ag*Δ*SHM*2-2 with [1-^{13}C]threonine in three independent experiments, depicted as fifth to seventh sets. The detected labelling patterns in threonine were set as 100% (shown in parentheses).

	^{13}C-labelling of C-1 (%)								
	Serine			Glycine			Threonine		
Strain	Fifth set	Sixth set	Seventh set	Fifth set	Sixth set	Seventh set	Fifth set	Sixth set	Seventh set
Wild-type	29.9 (31)	34.9 (35)	29.8 (31)	52.4 (54)	51.0 (52)	54.8 (57)	96.3 (100)	98.4 (100)	96.7 (100)
*Ag*Δ*SHM* 1	31.3 (34)	38.6 (40)		57.6 (60)	55.2 (57)		96.3 (100)	96.7 (100)	
*Ag*Δ*SHM* 2-1		5.3 (5)	4.2 (4)		65.5 (67)	56.1 (59)		97.7 (100)	94.8 (100)
*Ag*Δ*SHM* 2-2			1.5 (2)			59.2 (62)			95.1 (100)

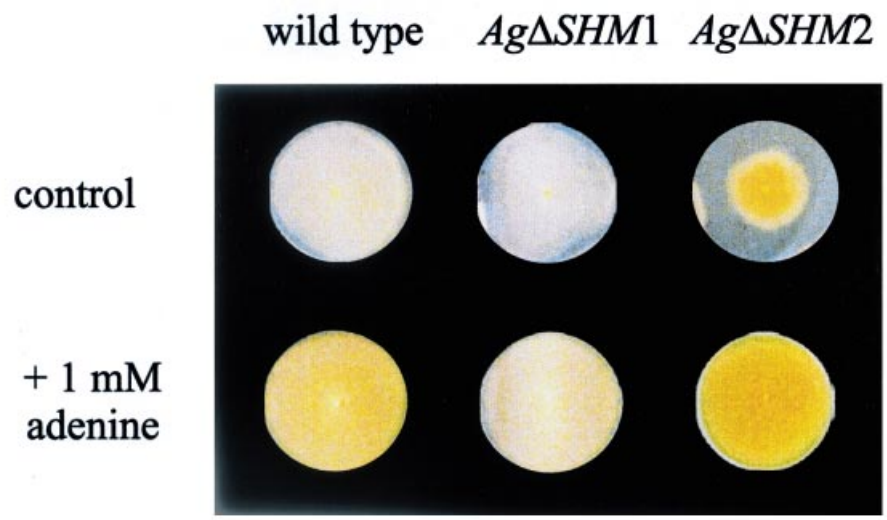


Figure 6 Effect of adenine supplementation on growth of *SHM* mutants

A. gossypii wild-type, *SHM*1- and *SHM*2-disrupted mutants were grown for 72 h on minimal medium with or without supplementation of 1 mM adenine.

Only 2, 4 and 5% were detected respectively. These labelling patterns in *Ag*Δ*SHM*2 correlate with the increased riboflavin overproduction, confirming the significant role of SHMT2 as a glycine-consuming enzyme.

Significance of AgSHMT1 and AgSHMT2 for growth

Besides the effects on riboflavin overproduction, the effects on growth were studied with or without supplementation of glycine, serine or threonine. If SHMTs contribute to the fluxes into the pools of these amino acids, complementation effects should be visible. Without amino acid supplementation (glycine $< 45\ \mu$M; serine $< 55\ \mu$M; and threonine $< 55\ \mu$M), *Ag*Δ*SHM*2 showed a reduced growth rate in comparison with the wild-type (Table 5). Although each amino acid had a positive effect on *Ag*Δ*SHM*2, e.g. 100 mM glycine increased the rate of growth from 2.3 mm/day to 3.4 mm/day, no amino acid supplementation was suitable to compensate this phenotype completely. This can be explained by a secondary effect causing growth inhibition. Growth inhibition of the wild-type was greatest with 100 mM serine, causing a complete lack of growth. Interestingly, *SHM* mutations increased the inhibition sensitivity in all but two cases. Feeding of 1 mM serine or threonine to *Ag*Δ*SHM*2 increased the growth rate. Wild-type growth could be restored by adding 1 mM adenine to *Ag*Δ*SHM*2 (Figure 6). The need for adenine to restore growth shows the linkage of serine and C_1 metabolism.

DISCUSSION

Supplementation of the medium with glycine is known to increase riboflavin overproduction in *A. gossypii* [6]. A greater effect on overproduction was found by an increase in intracellular glycine synthesis via overexpressed threonine aldolase [22]. Since this approach served as proof of the concept, SHMT has been considered to be an alternative glycinogenic enzyme. In *S. cerevisiae* and *C. albicans*, both closely related to *A. gossypii* [23], two *SHM* genes were identified encoding SHMT1 (which is localized in the mitochondria) and cytosolic SHMT2 [19,20]. We isolated two genes, *AgSHM*1 and *AgSHM*2, coding for *A. gossypii* SHMT isoenzymes. On account of the high levels of homology among the mitochondrial SHMT1s of *S. cerevisiae*, *C. albicans* and *Ag*SHMT1, and among the cytosolic SHMT2s of *S. cerevisiae*, *C. albicans* and *Ag*SHMT2, the probable localization of *Ag*SHMT1 and *Ag*SHMT2 is in the mitochondria and the cytosol respectively. This possible localization is confirmed by the identification of a pre-sequence at the N-terminus of SHMT1 that shows 61% identity with the mitochondria-targeting sequence.

To define the roles of the two SHMT isoenzymes in glycine metabolism in *A. gossypii*, *SHM*1 and *SHM*2 mutant strains were constructed and characterized. Disruption of *SHM*2 in *A. gossypii* led to an 85% loss of detectable SHMT activity, whereas *Ag*Δ*SHM*1 could not be distinguished from the wild-type level. A mutant overexpressing *SHM*1 under the control of the *TEF* promoter showed a 6-fold increase in SHMT specific activity, demonstrating both the functionality of SHMT1 and the relatively weak expression controlled by the original *SHM*1 promoter. These results are consistent with studies in yeast. *S. cerevisiae SHM*2-disrupted strains had 2–15% SHMT activity, compared with the parental strain, whereas the activity of the *SHM*1-disrupted strain was comparable with that of the parental strain, indicating that the cytosolic enzyme is the predominant SHMT in the yeast cell [19]. Separation of mitochondrial and cytosolic fractions showed that Δ*SHM*1 extracts lacked SHMT activity in the mitochondria fraction, whereas Δ*SHM*2 extracts lacked SHMT activity in the cytoplasmic fraction [19]. Further experiments by Kastanos et al. [24] confirmed these results and revealed that in *S. cerevisiae* wild-type only 5% of the total activity was mitochondrial. The *S. cerevisiae SHM*1-disrupted strain had wild-type levels of activity in the cytoplasm, but the specific activity in the mitochondria was less than 1% than that of the wild-type. On the other hand, *SHM*2-disrupted strains had activity levels comparable with wild-type in the mitochondria, but the cytoplasmic specific activity was less than 1% of the normal level. These data showed that the loss of one enzyme did not significantly affect the activity of the remaining isoenzyme.

In contrast with the disruption of *SHM*1, disruption of the *SHM*2 gene in *A. gossypii* led to a reduced growth rate in minimal medium. The addition of glycine improved the growth

of *SHM*2 mutants. In *S. cerevisiae*, inactivation of either one or both *SHM* genes did not result in a glycine requirement [19]. In contrast, bacterial strains deficient in SHMT exhibit an auxotrophy for glycine [25], whereas *Neurospora* strains lacking cytoplasmic SHMT require formate for growth [9]. Supplementation of 1 mM adenine restored the wild-type growth in *Ag*Δ*SHM*2. This was also observed by Piper et al. [26] in an *S. cerevisiae* ADE3 *shm*2 mutant strain, and shows that SHMT1 alone is not sufficient for a maximal one-carbon supply.

The contribution of each isoenzyme to the glycine metabolism was monitored by ^{13}C experiments. Supplementation with 1-^{13}C-labelled serine revealed a 6 % decrease in detected [1-^{13}C]glycine in *Ag*Δ*SHM*1, whereas in *Ag*Δ*SHM*2 a reduction of 37 % was observed. This indicates that both isoenzymes convert serine into glycine when serine is fed. Furthermore, it is obvious that SHMT2 can contribute more to glycine synthesis than SHMT1. [1-^{13}C]Threonine feeding showed that serine synthesis from glycine is not altered when *SHM*1 is disrupted. Concerning *Ag*Δ*SHM*2, threonine supplementation did not only lead to decreased labelling in the detected [1-^{13}C]serine, but also in an elevation of [1-^{13}C]glycine. This means, in the first instance, that the fed amino acids control the *in vivo* flux direction of SHMT, and secondly that SHMT2 is more affected because of its higher activity and cytosolic localization. When no amino acid is fed to *A. gossypii*, SHMT2 will surely work in the serinogenic direction, because the fungus is known to excrete glycine [22]. Consistently in *S. cerevisiae* [24], the nutritional conditions of the cell controlled the directions of the flux over the two isoenzymes, but, although a comparison of various experiments is always difficult, under conditions of glycine excess a serinogenic function of the cytosolic SHMT was not described [24].

In the *SHM*2-disrupted *A. gossypii* strains, labelling of glycine was increased, which correlates with the elevation of riboflavin overproduction. An explanation that connects the two results causally is that the inactivation of SHMT2 reduced the carbon flux from glycine towards serine, and therefore more glycine was available for riboflavin synthesis. Additionally, C_1 is saved, which is also needed for the synthesis of the riboflavin precursor GTP. Whether the C_1 supply limits riboflavin production cannot be yet established, since its metabolism is linked to glycine. One argument against a limitation of C_1 is that serine supplementation does not increase riboflavin production. A further clue is provided by the fact that Δ*SHM*2 strains produce more riboflavin, although their growth is limited by C_1, which was shown by the adenine supplementation.

A further interesting question is how the growth of *SHM*-disrupted strains is affected when glycine, serine or threonine is added. In eukaryotic cells, the study of growth effects of amino acids is complicated by the unequal distribution of amino acid pools between the cytosol and organelles. In *S. cerevisiae* [27] and *A. gossypii* [28], approx. 75 % of the intracellular glycine is located in the vacuole. Supplementation of $\geqslant 10$ mM glycine inhibited growth of *Ag*Δ*SHM*1. An increased cytosolic serine pool caused by SHMT2 could be a possible explanation for this effect. L-Serine has long been known to inhibit growth in *E. coli* cells. Hama et al. [29] concluded that the target of serine inhibition is homoserine dehydrogenase I. In *Ag*Δ*SHM*2 the growth rate increases when feeding $\geqslant 10$ mM glycine. In this case, the accumulation of serine is prevented because SHMT2 is inactivated. The beneficial effect of glycine, which might be a supply of the mitochondrially localized glycine-cleavage system for the liberation of one-carbon, becomes clear. Kastanos et al. [24] reported that in yeast the roles of SHMT isoenzymes vary, because the nutritional requirements of the cell vary. When serine is given in the *S. cerevisiae* medium, SHMT2 is the major provider of one-carbon units, whereas, when glycine is provided in the medium, the mitochondrial SHMT1 functions preferentially in the direction of serine synthesis. In *A. gossypii*, the opposite seems to be the case: supplementation of the medium with 1 mM serine improved the growth of *Ag*Δ*SHM*2, suggesting that SHMT1 had cleaved it to glycine and the needed one-carbon unit. The uptake of threonine by *A. gossypii* is facilitated compared with the glycine uptake [22]. Threonine is converted into glycine by threonine aldolase. The supplementation of threonine elevates the intracellular glycine pool more than the supplementation of glycine does [22]. In this regard, a roughly similar effect on growth is obtained when feeding 100 mM glycine or 1 mM threonine. The addition of 10 mM threonine decreased the growth rate in all strains, even in *Ag*Δ*SHM*2. Feedback inhibition of aspartate kinase, an important regulatory enzyme in the threonine-biosynthesis pathway, might provide an explanation. The threonine concentration for half-maximal inhibition of *S. cerevisiae* aspartate kinase is 3 mM [30]. A threonine-induced reduction of the carbon flow into the aspartate pathway can lead to methionine limitation, and subsequently to growth inhibition [31]. Supplementation of 10 mM serine resulted in a significant decrease in the growth rate in all three strains of *A. gossypii*, and the addition of 100 mM serine was completely toxic. This confirms the previous assumption that accumulation of serine within the cell leads to an inhibitory effect.

We are grateful to H. Sahm for helpful discussions and excellent working conditions. This work was supported by BASF AG.

REFERENCES

1 Vandamme, E. J. (1992) Production of vitamins, coenzymes and related biochemicals and related biotechnological processes. J. Chem. Tech. Biotechnol. **53**, 313–327
2 Stahmann, K.-P., Revuelta, J. L. and Seulberger, H. (2000) Three biotechnical processes using *Ashbya gossypii*, *Candida famata*, or *Bacillus subtilis* compete with chemical riboflavin production. Appl. Microbiol. Biotechnol. **53**, 509–516
3 Demain, A. L. (1972) Riboflavin oversynthesis. Annu. Rev. Microbiol. **26**, 369–388
4 Mehta, S. U., Mattoo, A. K. and Modi, V. V. (1972) Ribitol and flavinogenesis in *Eremothecium ashbyi*. Biochem. J. **130**, 159–166
5 Kaplan, L. and Demain, A. L. (1970) Nutritional studies on riboflavin overproduction by *Ashbya gossypii*. In Recent Trends in Yeast Research (Vol. 1) (Ahearn, D. G., ed.), pp. 137–159, Georgia State University, Atlanta
6 Hanson, A. N. (1967) Microbial production of pigments and vitamins. In Microbial Technology (Pepler, H. J., ed.), pp. 222–250, Reinhold, New York
7 Plaut, G. W. E. (1954) Biosynthesis of riboflavin II. Incorporation of ^{14}C-labelled compounds into ring A. J. Biol. Chem. **208**, 513–520
8 Mouillon, J.-M., Aubert, S., Bourguignon, J., Gout, E., Douce, R. and Rébeillé, F. (1999) Glycine and serine catabolism in non-photosynthetic higher plant cells: their role in C1 metabolism. Plant J. **20**, 197–205
9 McClung, C. R., Davis, C. R., Page, K. M. and Denome, S. A. (1992) Characterization of the formate (*for*) locus, which encodes the cytosolic serine hydroxymethyltransferase of *Neurospora crassa*. Mol. Cell. Biol. **12**, 1412–1421
10 Appling, D. R. (1991) Compartmentation of the folate-mediated one-carbon metabolism in eucaryotes. FASEB J. **5**, 2645–2651
11 Sambrook, J. and Russell, D. W. (2001) Molecular Cloning: a Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York
12 Sanger, F., Nicklen, S. and Coulson, A. R. (1977) DNA sequencing with chain terminating inhibitors. Proc. Natl. Acad. Sci. U.S.A. **74**, 5463–5467
13 Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D. J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25**, 3389–3402
14 Southern, E. M. (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**, 503–517
15 Schmidt, G., Stahmann, K.-P., Kaesler, B. and Sahm, H. (1996) Correlation of isocitrate lyase activity and riboflavin formation in the riboflavin overproducer *Ashbya gossypii*. Microbiology **142**, 419–426
16 Geller, A. M. and Kotb, M. Y. (1989) A binding assay for serine hydroxymethyltransferase. Anal. Biochem. **180**, 120–125
17 Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principal of protein dye-binding. Anal. Biochem. **72**, 258–264

18 Wendisch, V. F., de Graaf, A. A. and Sahm, H. (1997) Accurate determination of ^{13}C enrichments in nonprotonated carbon atoms of isotopically enriched amino acids by ^{1}H nuclear magnetic resonance. Anal. Biochem. **245**, 196–202
19 McNeil, J. B., McIntosh, E. M., Taylor, B. V., Zhang, F.-R., Tang, S. and Bognar, A. L. (1994) Cloning and molecular characterization of three genes, including two genes encoding serine hydroxymethyltransferase, whose inactivation is required to render yeast auxotrophic for glycine. J. Biol. Chem. **269**, 9155–9165
20 McNeil, J. B., Flynn, J., Tsao, N., Monschau, N., Stahmann, K.-P., Haynes, R. H., McIntosh, E. M. and Pearlman, R. E. (2000) Glycine metabolism in *Candida albicans*: Characterization of the serine hydroxymethyltransferase (*SHM1*, *SHM2*) and threonine aldolase (*GLY1*) genes. Yeast **16**, 167–175
21 Schirch, D., Delle Fratte, S., Iurescia, S., Angelaccio, S., Contestabile, R., Bossa, F. and Schirch, V. (1993) Function of the active-site lysine in *Escherichia coli* serine hydroxymethyltransferase. J. Biol. Chem. **268**, 23132–23138
22 Monschau, N., Sahm, H. and Stahmann, K.-P. (1998) Threonine aldolase overexpression plus threonine supplementation enhanced riboflavin production in *Ashbya gossypii*. Appl. Env. Microbiol. **64**, 4283–4290
23 Prillinger, H., Schweigkofler, W., Breitenbach, M., Briza, M., Staudacher, F., Lopandic, K., Molnar, O., Weigang, F., Ibi, M. and Ellinger, A. (1997) Phytopathogenic filamentous (*Ashbya*, *Eremothecium*) and dimorphic fungi (*Holleya*, *Nematospora*) with needle-shaped ascospores as new member within the Saccharomycetaceae. Yeast **13**, 945–960
24 Kastanos, E. K., Woldman, Y. Y. and Appling, D. (1997) Role of mitochondrial and cytoplasmic serine hydroxymethyltransferase isozymes in the *de novo* purine synthesis in *Saccharomyces cerevisiae*. Biochemistry **36**, 14956–14964
25 Stauffer, G. V., Plamann, M. D. and Stauffer, L. T. (1981) Construction and expression of hybrid plasmids containing the *Escherichia coli glyA* gene. Gene **14**, 63–72
26 Piper, M. D., Hong, S.-P., Ball, G. E. and Dawes, I. W. (2000) Regulation of the one-carbon metabolism in *Saccharomyces cerevisiae*. J. Biol. Chem. **40**, 30987–30995
27 Messenguy, F., Colin, D. and Ten Have, J. (1980) Regulation of compartmentation of amino acid pools in *Saccharomyces cerevisiae* and its effects on metabolic control. Eur. J. Biochem. **108**, 439–447
28 Förster, C., Marienfeld, S., Wilhelm, R. and Krämer, R. (1998) Organelle purification and selective permeabilisation of the plasma membrane: two different approaches to study vacuoles of the filamentous fungus *Ashbya gossypii*. FEMS Microbiol. Lett. **167**, 209–214
29 Hama, H., Sumita, Y., Kakutani, Y., Tsuda, M. and Tsuchija, T. (1990) Target of serine inhibition in *Escherichia coli*. Biochem. Biophys. Res. Commun. **168**, 1211–1216
30 Ramos, C., Delgado, M. A. and Calderon, I. (1991) Inhibition of different amino acids of the aspartate kinase and the homoserine kinase of the yeast *Saccharomyces cerevisiae*. FEBS **278**, 123–126
31 Arévalo-Rodríguez, M., Calderon, I. and Holmberg, S. (1999) Mutations that cause threonine sensitivity identify catalytic and regulatory regions of the aspartate kinase of *Saccharomyces cerevisiae*. Yeast **15**, 1331–1345

Received 5 August 2002/23 September 2002; accepted 26 September 2002
Published as BJ Immediate Publication 26 September 2002, DOI 10.1042/BJ20021224