Threonine Aldolase Overexpression plus Threonine Supplementation Enhanced Riboflavin Production in Ashbya gossypii

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Riboflavin production in the filamentous fungus Ashbya gossypii is limited by glycine, an early precursor required for purine synthesis. We report an improvement of riboflavin production in this fungus by overexpression of the glycine biosynthetic enzyme threonine aldolase. The GLY1 gene encoding the threonine aldolase of A. gossypii was isolated by heterologous complementation of the glycine-auxotrophic Saccharomyces cerevisiae strain YM13 with a genomic library from A. gossypii. The deduced amino acid sequence of GLY1 showed 88% similarity to threonine aldolase from S. cerevisiae. In the presence of the GLY1 gene, 25 mU of threonine aldolase specific activity mg⁻¹ was detectable in crude extracts of S. cerevisiae YM13. Disruption of GLY1 led to a complete loss of threonine aldolase activity in A. gossypii crude extracts, but growth of and riboflavin production by the knockout mutant were not affected. This indicated a minor role of the enzyme in glycine biosynthesis of A. gossypii. However, overexpression of GLY1 under the control of the constitutive TEF promoter and terminator led to a 10-fold increase of threonine aldolase specific activity in crude extracts along with a 9-fold increase of riboflavin production when the medium was supplemented with threonine. This strong enhancement, which could not be achieved by supplementation with glycine alone, was attributed to an almost quantitative uptake of threonine and its intracellular conversion into glycine. This became evident by a subsequent partial efflux of the glycine formed.

The filamentous hemiascomycete Ashbya gossypii (Ashby and Novell) (10) is a biotechnologically important producer of vitamin B₂ (riboflavin). Improved producer strains are used for commercial production of riboflavin (7) and give a yield of up to 15 g liter⁻¹ (4). Since the first quantitative report (45), there have been numerous efforts to improve riboflavin production by A. gossypii by optimization of medium composition and fermentation conditions (20, 27) as well as by screening of antimetabolite-resistant mutants (38, 39). Since recombinant DNA techniques for A. gossypii have been established recently (42, 46), the way is now open for development of a targeted producer strain by molecular approaches.

Starting from GTP and ribulose-5-phosphate only six enzymatic reactions are specific for the biosynthetic pathway of riboflavin (3). However, metabolic flux to riboflavin is also determined by numerous nonspecific reactions providing sufficient amounts of the two starting metabolites. This is indicated by the observation that riboflavin production can be enhanced by supplementation of the culture medium with different riboflavin precursors, e.g., ribitol (24), purines (18), and glycine (7, 12, 18). The yield-enhancing effect of glycine, which is an important precursor during de novo purine biosynthesis, has also been described for riboflavin production by Candida flareri (13). Quantitative incorporation of glycine into the riboflavin molecule was already reported by Plaut in 1954 (29). It is mediated by glycine amid ribonucleotide synthetase, which catalyzes the formation of an amido linkage between glycine and 5-phosphoribosylamine, consuming one molecule of ATP. Against this background, the present study aimed at the improvement of fungal glycine biosynthesis, leading to a better precursor supply of purine and subsequent riboflavin formation.

Three main routes of glycine biosynthesis have been described so far. Among plants, animals, and microorganisms the most widespread glycine biosynthetic enzyme is serine hydroxymethyltransferase (SHMT; EC 2.1.2.1), which catalyzes the tetrahydrofolate-dependent cleavage of serine into glycine and 5,10-methylene-tetrahydrofolate. This reaction is the only one producing glycine in Escherichia coli (40). Accordingly, inactivation of the glyA gene, encoding SHMT, leads to glycine auxotrophy in E. coli. In Saccharomyces cerevisiae, this pathway of glycine biosynthesis is termed the glycolytic pathway, as it starts from the glycolytic intermediate 3-phosphoglycerate. It is contrasted with the gluconeogenic pathway, which starts from glyoxylate, a product of the anaplerotic glyoxylate cycle. Synthesis of alanine glyoxylate aminotransferase (EC 2.6.1.44), the key enzyme of the latter pathway, is subject to glucose repression, so that the gluconeogenic pathway is the major source of glycine only during growth of S. cerevisiae on nonfermentable carbon sources such as ethanol and acetate (43). Recently, this model of glycine biosynthesis in yeast has been expanded by a threonine aldolase (EC 4.1.2.5), which provides a significant amount of glycine during growth of S. cerevisiae on glucose (22, 25). Growth studies even suggested that threonine aldolase is the major source of glycine under these conditions, since disruption of the corresponding gene (GLY1) led to a strongly reduced growth rate in the absence of glycine. However, disruption of both SHM genes, encoding the two SHMT isoenzymes in yeast, did not significantly affect the growth rate (23).

The present work aimed at improving riboflavin production in A. gossypii by enhancing the biosynthesis of the riboflavin precursor glycine. Cloning, disruption, and overexpression of the A. gossypii GLY1 gene, encoding a threonine aldolase, are reported. Evidence is presented that overexpression of this

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glycine biosynthetic gene can lead to an increase in riboflavin production.

MATERIALS AND METHODS

Chemicals. Lysing enzymes from Trichoderma harzianum were purchased from Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany. [3-4C]Cerine was supplied by Amersham Buchler GmbH and Co. KG, Germany.

Strains and growth conditions. A. gossypii ATCC 10895 was used as an A. gossypii type strain. The A. gossypii YM13 [ATCC 28077; MTAX ura-3 tspl-1 adel-2 his-3-115 leu-2-3,112 can-1 100 shml-1: HIS3 shml-2: LEU2 g31:URA3] (23) and its uracil auxotrophic derivative YM13F (see below) were used for complementation experiments with the A. gossypii genomic library. E. coli DH5α [F- lacZAM15 Δ(lac-proAB) recA1 endA1 gal-46 thi-1] (11) was the recipient for plasmid amplification.

A. gossypii strains were maintained on solid complex medium (1% yeast extract, 1% glucose). For selection and selective growth of transformants, Geneticin-resistance plasmids were introduced into partly digested with A. gossypii YEp352 (15). Chromosomal DNA isolated from S. cerevisiae—E. coli shuttle vectors was grown in liquid medium supplemented with 50 

Transformation of A. gossypii. Transformation of A. gossypii was done by an electroporation method developed by Revuelta (32a). Fungal mycelium was grown overnight in liquid complex medium, harvested by filtration, washed with the electroporation buffer, and stored at 

Sequence determination and alignment. The GLY1 nucleotide sequence was determined on both strands by the dyeosequencing technique of Sanger et al. (34). Homology searches were performed by using the CLUSTALW similarity search program BLAST (28). The CLUSTAL method (14) was used for multiple alignment of sequences.

Gene disruption. GLY1 knockout mutants were constructed by replacing the 5'-terminal 500 bp of the GLY1 coding sequence by a kanamycin-resistance cassette. The 5.7-kbp HindIII fragment from the subclone YEp352 GB 26-9-6, which carries the GLY1 gene (except the 3'-terminal 48 bp) and 2.7 kb upstream of the gene, was inserted into pUC18 (44) at the HindIII site. The resulting plasmid, pUC1GGLY1, was used as a template in a PCR with the primers GLY1UP (5'-CTTCTGCAAGACGCTTGGATAGGACG-3') and GLY1DOWN (5'-CTTGCAGAAGGCTTGGTATGGGAGAAC-3'), resulting in amplification of the whole plasmid except for the 5'-terminal 500 bp of the GLY1 gene. At the underlined position of each primer, an XhoI site was introduced so that the PCR product could be religated after XhoI cleavage (pUC18GLY1/2400). A 1.6-kb Safl fragment of plasmid pAG231 (42), which carried the E. coli kanamycin resistance gene under the control of the A. gossypii TEF promoter and terminator (TATTTTA GTTATTTTA), was subcloned into pUC18GLY1XhoI and inserted into the XhoI site of pUC18GLY1D500. The kanamycin resistance gene can be used as a selectable marker conferring resistance to Geneticin in eukaryotes (16). The resulting plasmid pUC1Ggly1/kan (see Fig. 3a) was digested with XhoI and used to transform A. gossypii. Screening of transformants allowed to construct an autonomous replicative sequence for A. gossypii and homologous recombination is described as the main mechanism for DNA integration in A. gossypii (12), disruption of GLY1 was expected in all Geneticin-resistant transformants.

Construction of the GLY1 overexpression plasmid pAG203GLY1. PCR amplification of the GLY1 gene was carried out to permit cloning into the expression plasmid pAG203 (19). It uses the TEF promoter for a strong and constitutive expression. The primers GLY1UP (5'-AACTCAGATTAAAGCTTGTAGTCAACTGTAGCAG-3') and GLY1DOWN (5'-ATACCCGATCAGATAGCCAACTGTTAGTCAACTGTAGCAG-3') were designed on the basis of the nucleotide sequence of the GLY1 gene. To facilitate cloning, additional restriction sites (Spfl and Hpal, respectively; exchanged nucleotides are underlined) were introduced into each primer. Introduction of the Spfl site into primer GLY1-START was expected to enable the cloning in frame to the ATG initiation codon available on the plasmid pAG203 and led to a modification of the second codon from AAT to CAA. This corresponds to the conserved amino acid exchange Asn—Gln. The amplified PCR product was digested with Spfi and Hpal, purified by agarose gel electrophoresis, and inserted into Spfl-ScaI-linearized pAG203. The resulting plasmid was designated pAG203GLY1.

Determination of total riboflavin dry weight. For the determination of total riboflavin, cells were disrupted by the addition of 100 

Cell extraction. A. gossypii mycelium was harvested by filtration, rinsed with distilled water, and resuspended in 50 mM HEPES—NaOH buffer (pH 7.0)–1 mM DTT and 0.25 mg of protein (from crude extracts). The labeled 5,10-methylene
tetrahydrofolate was separated from unreacted serine by streaking 20-

Enzyme assays. Threonine aldolase activity was determined by quantification of the glycine formed in a high-pressure liquid chromatography system as previously described (25). In a final volume of 250 

Protein concentrations were determined spectrophotometrically by the

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FIG. 1. Schematic representation of the sequenced genomic DNA insert. The positions of the two ORFs GLY1 and ORF1 are indicated by black arrows. The solid bars above the map represent the sequences present in the relevant genomic clones YEp352 GB 26-9 and YEp352 GB 7-1 and in the subclone YEp352 GB 26-9-6. The 3′-terminal HindIII sites in YEp352 GB 26-9 and YEp352 GB 26-9-6 originate from the multiple-cloning site (MCS) of the cloning vector YEp352. A 941-bp SacI-MnNI fragment that was used as a probe for Southern hybridization is indicated by the shaded bar.

method of Bradford (6) at 595 nm with the Serva Blue G dye binding reagent. Bovine serum albumin was used as a standard.

Nucleotide sequence accession number. Sequence data have been submitted to the EMBL database and are listed under accession no. AJ005442.

RESULTS

Cloning and sequencing of the A. gossypii GLY1 gene. To isolate the GLY1 gene from A. gossypii, heterologous complementation of the glycine auxotrophic yeast strain YM13 (shm1:: HIS3 shm2::LEU2 gly1::URA3) with a plasmid library of genomic A. gossypii DNA in YEp352 (Amp′ URA3) was performed. However, no transformants could be isolated by direct selection for glycine prototrophy. Therefore, uracil auxotrophic mutants of the yeast strain YM13 were isolated by selection for 5-FOA resistance (for details, see Materials and Methods) to allow preselection of plasmid-containing clones. One of the spontaneous 5-FOA-resistant mutants (YM13F) was subsequently used for a second transformation with the genomic library. Preselection for uracil prototrophy led to the isolation of 70,000 transformants, which were replica plated on minimal medium without glycine. From this medium, 25 glycine-prototrophic clones were isolated. Curing from the plasmid by two subsequent cultivations on complex medium as well as retransformation with the isolated plasmids showed that complementation was linked to uracil prototrophy, which indicated that it was due to a cloned fragment. Restriction analysis of the isolated plasmids showed that they carried inserts of 10 to 12 kb, all of them containing the same gene. Subcloning proceeding from the genomic clone YEp352 GB 26-9 revealed complementation of a 3.7-kb HindIII fragment (YEp352 GB 26-9-6), which was subsequently sequenced. It carried two incomplete open reading frames (ORFs), 1,326 bp of ORF1 and 1,098 bp of ORF2 (Fig. 1). The deduced amino acid sequence of ORF1 showed 88% similarity to threonine aldolase (GLY1) from S. cerevisiae. The predicted amino acid sequence of ORF1 displayed 54% similarity to YEL043w, a hypothetical 106.1-kDa protein of unknown function encoded in the GLY1-GDA1 intergenic region of S. cerevisiae. The striking similarity of ORF2 to the S. cerevisiae GLY1 gene, together with the detection of 25 mU of threonine aldolase specific activity mg of protein−1 in the yeast transformants, in contrast to <0.1 mU mg of protein−1 in the control (25), led to the conclusion that a GLY1 homologue had been isolated from A. gossypii. The full sequence of the A. gossypii GLY1 gene was subsequently determined by partial sequencing of the genomic clone YEp352 GB 7-1 (Fig. 1). The A. gossypii GLY1 gene has an ORF of 1,146 bp encoding a predicted protein of 382 amino acids.

Alignment of A. gossypii threonine aldolase with other proteins. A multiple amino acid sequence alignment that includes threonine aldolase from A. gossypii and other proteins whose sequences have previously been reported is shown in Fig. 2. Apart from yeast threonine aldolase, with 88% similarity, the putative A. gossypii protein showed the highest similarity to two other fungal proteins, namely, threonine aldolase from Candida albicans (76%) and a hypothetical protein from Schizosaccharomyces pombe (62%). We also detected high similarity to a protein from the bacterium Aeromonas jandiae (62%), which was recently identified as an 1-alle-threonine aldolase (21), as well as to a hypothetical protein from the nematode Caenorhabditis elegans (54%). Apart from the C. elegans protein, all six proteins are of similar length. Whereas the C and N termini of the proteins are only weakly conserved, the central part contains two highly conserved domains. One of them contains Lys199 of the 1-alle-threonine aldolase of Aeromonas jandiae, which was recently identified as the pyridoxal 5′-phosphate binding lysine residue essential for the aldol cleavage reaction by site-directed mutagenesis (21). This residue is conserved among all six proteins. Threonine aldolase from Aeromonas jandiae is by far the smallest of the six proteins, lacking the weakly conserved C- and N-terminal regions. This indicates that these residues might not be important for the enzymatic activity of the protein and explains why the formation of a truncated protein from plasmids YEp352 GB 26-9 and YEp352 GB 26-9-6 led to complementation of glycine auxotrophy in S. cerevisiae YM13.

Characterization of GLY1 knockout mutants. The chromosomal GLY1 gene was inactivated by gene disruption. A linearized DNA fragment containing the Geneticin resistance cassette flanked by the 3′ end of the GLY1 gene on one side and by the upstream region of GLY1 on the other side was used to transform A. gossypii ATCC 10895 to Geneticin resistance (Fig. 3a). Gene disruption was confirmed by PCR (Fig. 3b). As expected, a 1.3-kb shift of the signal was observed. Beyond that, Southern hybridization (Fig. 3c) demonstrated that replacement with the resistance marker had occurred at the GLY1 locus.

Inactivation of GLY1 resulted in a complete loss of detectable threonine aldolase activity (<0.1 mU mg of protein−1). This indicated that GLY1 is the only gene encoding a threonine aldolase in A. gossypii. However, SHMT specific activity still reached the wild-type level (2.5 to 3 mU mg of protein−1), which demonstrated that this reaction is catalyzed by a different enzyme in A. gossypii. Disruption of GLY1 did not lead to a requirement for glycine. Growth and riboflavin production on minimal medium without supplementary glycine turned out to be unchanged in the GLY1 disruption mutants.

Overexpression of GLY1. Although disruption of GLY1 had demonstrated that threonine aldolase is probably not essential for glycine biosynthesis in the A. gossypii wild-type strain, overexpression of the gene seemed to be promising with regard to an improvement of glycine biosynthesis. Overexpression of the GLY1 gene was achieved by introducing the expression plasmid pAG203GLY1 (see Materials and Methods) into A. gossypii ATCC 10895. Transformants showed a ca. 10-fold increase in threonine aldolase specific activity over the whole time course of cultivation (Fig. 4a). The growth remained unchanged (Fig. 4b). However, it was not possible to obtain a constant level of threonine aldolase specific activity throughout cultivation. As in the control strain, which was transformed only with the control plasmid pAG203, enzyme activity decreased drastically from 12 to 34 h of cultivation. To ensure
that this decrease was not due to a decrease of promoter activity, β-galactosidase was expressed under the control of the TEF promoter by using the plasmid pAG110 (41). In this case, 70% of the starting activity was still detectable at the end of the cultivation time (data not shown).

As shown in Fig. 4c, overexpression of threonine aldolase alone did not lead to an improved riboflavin production in this experiment. Therefore, riboflavin production was subsequently investigated on complex medium supplemented with glycine or threonine. Figure 5 demonstrates that supplementation with 80 mM glycine led to an about threefold increase in riboflavin production in the wild-type strain as well as in the two plasmid-containing strains of A. gossypii pAG203 and A. gossypii pAG203 GLY1. However, if the culture medium was supple-
mented with 50 mM threonine, an eightfold increase in riboflavin production was detectable in \textit{A. gossypii} pAG203GLY1 whereas threonine did not affect riboflavin production in the two control strains. When the amino acid concentrations of the culture medium were determined after cultivation, it became apparent that in strain \textit{A. gossypii} pAG203GLY1, the threonine concentration had dropped from 50 ± 5 to 6 ± 0.5 mM and simultaneously the glycine concentration had increased from 2 ± 0.2 to 41 ± 4 mM. In the control strain \textit{A. gossypii} pAG203, the decrease in threonine concentration (from 50 ± 5 to 32 ± 3 mM) and the increase in glycine concentration (from 2 ± 0.2 to 6 ± 0.5 mM) were much smaller. Thus, the yield-enhancing effect of threonine aldolase overexpression plus threonine supplementation must be attributed to an increased uptake of

**FIG. 3.** Disruption of the \textit{GLY1} gene. (a) Schematic representation of the disruption construct pUC18\textit{gly1::kan} and the single-step gene disruption procedure. The intact genomic \textit{GLY1} gene was replaced by an incomplete copy together with the \textit{Kan} gene by means of a double crossover. On the \textit{XbaI} fragment, DNA regions identical to the chromosome are shaded. The numbers refer to the start of the \textit{GLY1} gene. (b and c) Disruption of the gene was verified by PCR (b) and Southern hybridization (c) in the knockout mutants \textit{ΔGLY1-1}, \textit{ΔGLY1-2}, and \textit{ΔGLY1-3}. For Southern analysis, genomic DNA was digested simultaneously with \textit{BamHI} and \textit{MluI}, as indicated, and a 941-bp \textit{SacI-MluI} fragment was used as a probe (Fig. 1). For PCR, the primers INTEGRA-UP (5'-AGGAGCGTTACGTCCAACGTCGTTCTGTG-3') and INTEGRA-DOWN (AAATGGTAGAGCTACTAGCCCTCCGCAATA-3'), which were derived from sequences upstream and downstream, respectively, of the \textit{GLY1} gene, were used.
extracellular threonine and its intracellular conversion into glycine. The weaker effect of glycine supplementation could be due to a lower uptake: this amino acid concentration decreased only slightly from 82 ± 2 to 79 ± 1 mM.

**DISCUSSION**

Threonine aldolase is thought to be the major source of glycine in the yeast *S. cerevisiae* (23, 25). Therefore, it was a promising candidate for the improvement of glycine biosynthesis and subsequent riboflavin production in the closely related filamentous fungus *A. gossypii*.

The ORF isolated by heterologous complementation of the glycine-auxotrophic *S. cerevisiae* strain YM13 was identified as encoding threonine aldolase of *A. gossypii* by detection of the enzymatic activity of the corresponding protein in crude extracts as well as by an amino acid alignment with threonine aldolases from *S. cerevisiae*, *C. albicans*, and Aeromonas jandiae. The remarkably high homology (88%) between the deduced amino acid sequences of the two threonine aldolases from *A. gossypii* and *S. cerevisiae* underlines the close relationship between the two fungi, which was concluded from an alignment of ITS1 and ITS2 sequences by Prillinger et al. (31) and led to a new definition of the family Saccharomycetaceae that included both unicellular saprophytic yeasts and dimorphic or filamentous parasitic fungi. Furthermore, colocation of the GLY1 gene and a YEL043w homologue in *A. gossypii* is another good example of conservation of gene order in *A. gossypii* and *S. cerevisiae*. Only recently was the *A. gossypii* THR4 gene located in a four-gene cluster that is conserved between *A. gossypii* and *S. cerevisiae* (1).

Surprisingly, heterologous complementation of *S. cerevisiae* YM13 did not lead to the isolation of a gene encoding SHMT. This is probably due to the particular importance of threonine aldolase for glycine biosynthesis in yeast. We had already reported in a previous paper (25) that growth of *S. cerevisiae* YM13 can be completely restored by transformation with a plasmid containing the GLY1 gene. On the other hand, McNeil et al. (23) demonstrated that both gly1 shm1 and gly1 shm2 double mutants are severely impaired in growth. Taking into consideration that heterologous expression of an SHM gene from *A. gossypii* in yeast is probably even weaker than that of the homologous gene, it becomes conceivable that this might not be sufficient to permit growth of the glycine-auxotrophic yeast strain.

Disruption of GLY1 in *A. gossypii* led to a complete loss of detectable threonine aldolase activity, whereas SHMT still reached the wild-type level. This indicated that the aldol cleavage reaction of serine into glycine and 5,10-methylene tetrahydrofolate is catalyzed by a separate enzyme in *A. gossypii*. This is in agreement with the situation in yeast (23) but contrasts with that in *E. coli* (36) and rat liver (35), where threonine aldolase activity was demonstrated for purified SHMT. In *S. cerevisiae*, disruption of the GLY1 gene leads to a strongly reduced growth rate in the absence of glycine whereas the disruption of both SHM genes does not (23). These studies suggested that threonine aldolase is the major source of glycine.
in yeast. In contrast, disruption of GLY1 did not lead to a requirement for glycine in A. gossypii. Additionally, a decrease in riboflavin production, indicating a reduced glycine supply, was not detectable. That means that threonine aldolase plays only a minor role during glycine biosynthesis of A. gossypii or that its function can be fully compensated by other glycine biosynthetic pathways.

A ca. 10-fold increase in threonine aldolase specific activity was reached by overexpression of GLY1 under the control of the TEF promoter and terminator. However, there was still a drastic decrease in activity during the course of cultivation. Although TEF promoters from different sources have been shown to be comparatively strong (2, 37), activity was demonstrated to depend on the age of the tissue for the TEF promoter from Lycopersicon esculentum (30). Such a promoter-dependent kind of regulation can be excluded in our case, because β-galactosidase, expressed under the control of the TEF promoter, did not show a similar time course. Consequently, the decrease in threonine aldolase specific activity must be attributed to a promoter-independent type of regulation on the mRNA or protein level. Apart from these in vitro data, overexpression of threonine aldolase was also demonstrated in vivo by the almost quantitative conversion of extracellular threonine into glycine in the overexpression strain A. gossypii pAG203GLY1.

Overexpression of GLY1, together with threonine supplementation of the culture medium, led to a strong enhancement of riboflavin production, which could not be achieved by glycine supplementation alone. From the almost quantitative conversion of the extracellular threonine into glycine, we conclude that this enhancement must be due to an increased uptake of extracellular threonine and its subsequent intracellular conversion into glycine. Although most of the glycine produced was subsequently excreted into the medium, this obviously improved the intracellular availability of glycine and led to an enhanced riboflavin production. The finding that improvement of riboflavin production requires both overexpression of threonine aldolase and threonine supplementation leads to the conclusion that threonine biosynthesis must be the limiting factor under these conditions. We have evidence that feedback inhibition of aspartokinase, an important regulatory enzyme during threonine biosynthesis in S. cerevisiae (32) and Corynebacterium glutamicum (17), is responsible for this limitation. Elimination of this feedback inhibition could therefore be the key to further improvement of riboflavin production without any amino acid supplementation of the culture medium.

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