A Chemogenomic Screening of Sulfanilamide-Hypersensitive Saccharomyces cerevisiae Mutants Uncovers ABZ2, the Gene Encoding a Fungal Aminodeoxychorismate Lyase

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Large-scale phenotypic analyses have proved to be useful strategies in providing functional clues about the uncharacterized yeast genes. We used here a chemogenomic profiling of yeast deletion collections to identify the core of cellular processes challenged by treatment with the p-aminobenzoate/folate antimetabolite sulfanilamide. In addition to sulfanilamide-hypersensitive mutants whose deleted genes can be categorized into a number of groups, including one-carbon related metabolism, vacuole biogenesis and vesicular transport, DNA metabolic and cell cycle processes, and lipid and amino acid metabolism, two uncharacterized open reading frames (YH19 and YMR289w) were also identified. A detailed characterization of YMR289w revealed that this gene was required for growth in media lacking p-aminobenzoic or folic acid and encoded a 4-amino-4-deoxychorismate lyase, which is the last of the three enzymatic activities required for p-aminobenzoic acid biosynthesis. In light of these results, YMR289w was designated ABZ2, in accordance with the accepted nomenclature. ABZ2 was able to rescue the p-aminobenzoate auxotrophy of an Escherichia coli pabC mutant, thus demonstrating that ABZ2 and pabC are functional homologues. Phylogenetic analyses revealed that Abz2p is the founder member of a new group of fungal 4-amino-4-deoxychorismate lyases that have no significant homology to its bacterial or plant counterparts. Abz2p appeared to form homodimers and dimerization was indispensable for its catalytic activity.

The study of the folate biosynthetic pathway has been restricted to a few species of bacteria and plants (4, 5, 15). The folate molecule is tripartite, comprising of pteridine, glutamate, and p-aminobenzoate (PABA) moieties. In Escherichia coli, folic acid is biosynthesized by coupling of PABA and 7,8-dihydro-6-hydroxymethylpterin pyrophosphate to produce 7,8-dihydropteroylglutamate, which is subsequently glutamylated to give 7,8-dihydrofolate and reduced to yield tetrahydrofolate. The PABA moiety is synthesized in two steps catalyzed by two separate enzymes (Fig. 1). In bacteria, aminodeoxychorismate synthase (a heterodimeric enzyme formed by the association of the subunits encoded in E. coli by pabA and pabB) synthesizes 4-amino-4-deoxychorismate (ADC) from chorismate and glutamine (15). However, the pathway leading to PABA in Saccharomyces cerevisiae has not yet been completely elucidated. A bifunctional gene (ABZ1) encodes a protein bearing similarity to the two components (PabA and PabB) of ADC synthase described for E. coli (9). However, the gene that presumably encodes ADC lyase in yeast remains to be identified. Based on a large-scale screening of yeast mutants hypersensitive to the toxic PABA-analogue sulfanilamide, we identified a gene of previously unknown function (YMR289w/ABZ2) that is required for the biosynthesis of PABA. Here we show that the enzyme encoded by ABZ2 is needed to convert ADC to PABA. On the basis of sequence similarity, the product of ABZ2 is representative of a group of relatively homologous ADC lyases from fungi that are distantly related to the prokaryotic and plant enzymes. This finding completes the identification of the 10 enzymatic steps that convert PABA and GTP into tetrahydrofolate in the pathway for vitamin B9 biosynthesis in yeast.

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FIG. 1. Biosynthesis of PABA in E. coli and in S. cerevisiae. Names in italics show the E. coli genes that encode the enzyme. PabA and PabB associate to form the ADC synthase complex. Name in italics and in parentheses represents the corresponding S. cerevisiae gene; ABZ1 encodes a bifunctional PabA-PabB ADC synthase. pABA, PABA.

MATERIALS AND METHODS

Yeast strains and growth conditions. The collection of nonessential haploid MATα yeast deletion strains derived from parental strain BY4742 (MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0) was obtained from Euroscarf (Frankfurt, Germany; http://web.uni-frankfurt.de/fb15/mikro/euroscarf/index.html), while the essential haploid yeast strains were derived from the yeast knockout collection. Strains with enhanced sensitivity to sulfanilamide were selected based on their optical density at 595 nm, using a microplate reader spectrophotometer (model 550; Bio-Rad Laboratories). Putative sulfanilamide-hypersensitive mutants were identified during the screening of the yeast knockout collection were then retested at least in duplicate under the same conditions described for the yeast strains identified during the screening of the yeast knockout collection.

Large-scale sulfanilamide sensitivity screenings. Approximately 4,800 haploid deletion strain collections were generated by the Genome Deletion Project (http://www-sequence.stanford.edu/group/yeast_deletion_project/deletions3.html) by replacing the target gene with a kanamycin resistance cassette, KanMX4, by means of a PCR-based gene disruption strategy (44). Yeast strains were grown on standard rich medium YPD (1% yeast extract, 2% Bacto peptone, 2% glucose) and synthetic minimal medium (SMM) (43) supplemented with the nutritional requirements of the parental strain. Agar (2%) was added for solid plates. All yeast cultures were incubated at 28°C.

Enzyme assays. Stock solutions (5 mM) of chorismic acid (Sigma) were extracted three times with diethyl ether to remove contaminating 4-hydroxybenzoate. l-Glutamine and PABA, obtained from Sigma, were used without any further purification. ADC lyase activity was determined using purified yeast recombinant ADC lyase precluded by treatment, or 75%. The GI was calculated according to the following equation: GI = 100 × [(growth of mutant in control medium – growth of mutant in drug-containing medium)/growth of mutant in control medium].

Expression and purification of Abz2p. To express the full-length Abz2p protein, a plasmid was generated by inserting the S. cerevisiae ABZ2 coding sequence (PCR amplified) between the NdeI and BamHI cloning sites of the pET-28b expression plasmid (Novagen). In the recombinant vector (pET28b-Abz2), the Abz2 polypeptide is fused in frame with an N-terminal peptide containing six tandem histidine residues, and expression of the His-tagged protein is driven by a T7 RNA polymerase promoter. The DNA sequence of the insert was confirmed.

The pET28b-Abz2 recombinant plasmid was transformed into E. coli BL21(DE3), and a 1,000-ml culture of E. coli BL21(DE3)/pET28b-Abz2 was grown at 37°C in Luria-Bertani medium containing 50 μM pyridoxal phosphate and 30 μg of kanamycin/ml until achieving an A600 of 0.8. The culture was adjusted to 0.4 mM IPTG (isopropylβ-D-thiogalactoside), and incubation was continued at 18°C for 20 h. The cells were then harvested by centrifugation, and the pellet was stored at −80°C. All subsequent procedures were performed at 4°C. Thawed bacterial pellets were resuspended in 50 ml of lysis buffer A (50 mM potassium phosphate buffer [pH 7.5], 500 mM NaCl, 20 mM imidazole, 0.01 M β-mercaptoethanol, protease inhibitor coil) (Rochi Diagnostics), and cell lysis was achieved by the addition of 1 mg of lysozyme/ml. The lysates were sonicated to reduce viscosity, and any insoluble material was removed by centrifugation at 15,000 × g for 45 min.

The soluble extract was applied to a 5-ml Ni-affinity column (HisTrap HP; GE Healthcare) that had been equilibrated with buffer A. Fractions from MonQ chromatographies containing apparently pure Abz2p were pooled and desalted on a PD-10 column, and glycerol was added (10% [vol/vol] final concentration) prior to storage at −80°C. The purity of the protein was checked by sodium dodecyl-sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (26). The protein concentration was determined by the Bio-Rad dye binding method, using bovine serum albumin as standard (Bio-Rad).

Enzyme assays. Stock solutions (5 mM) of chorismic acid (Sigma) were extracted three times with diethyl ether to remove contaminating 4-hydroxybenzoate. l-Glutamine and PABA, obtained from Sigma, were used without any further purification. ADC lyase activity was determined using purified yeast recombinant ADC lyase precluded by treatment, or 75%. The GI was calculated according to the following equation: GI = 100 × [(growth of mutant in control medium – growth of mutant in drug-containing medium)/growth of mutant in control medium].
TABLE 1. Functional classification of genes whose deletion causes hypersensitivity to sulfanilamide

| Function                              | No. of genes | Mutants hypersensitive to sulfanilamide* |
|---------------------------------------|--------------|-----------------------------------------|
| One-carbon metabolism                 | 17           | ABZ1, ADK1, ADE6, AR01, CKB1, CKB2, FOL2*, GSH1, GUK1, HRT1, MET6, MET7, MET8, MET30, PHO2, RIB3, RM7 |
| Amino acid metabolism                 | 8            | BRO1, CCR4, LST8, NOT5, PTR3, RTG1, TRP5, URE2 |
| Lipid metabolism                     | 16           | ARG82, DEP1, EER4, ERG6, ERG8, FAB1, GPH1, HEM14, MVD1, NSG2, OPI1, PHO33, RX2, SAP30, UME6, VPS34 |
| Vacuole and vesicular transport       | 24           | CHC1, PEP3, PEP5, RGP1, SEC12, SLA2, TFP1, TPP3, TSA1, VMA4, VMA5, VMA7, VMA8, VMA10, VMA13, VMA16, VMA21, VMA22, VPH2, VPS16, YPS4, YPS52, YPS54, YPR1 |
| DNA metabolism and cell cycle         | 28           | ARP5, ARP8, CAK1, CDC2, CDC8, FUR4, FYV6, GCN5, GRR1, HIS2, INO80, IR1, KEM1, MCI1, POL32, RAD52, RFC2, RNR2, RRM3, RSC6, RTT109, SGF29, SNE2, SPC72, SPT3, STH1, SWD1, YBI1 |
| Biological process unknown            | 6            | OP9, YBL100C, YCL007C, YH9, YMR289W, YOR331C |
| Other functions                       | 18           | BCY1, SGAI, SHP1, TPS2 |
| Carbohydrate metabolism              |              | ANP1, KRE2, MNN9, OCHI |
| Mannosylation                         |              | HPR1, RLR1, RPB4, SNF4, SUB1, THP1, TUP1 |
| Transcription                         |              | AAC3, ATP1, ATP2 |
| Energy                                |              | |

* Genes identified among the essential heterozygous diploids set are indicated in boldface.

the molecular mass of the Abz2 polypeptide under denaturing conditions. Identification of proteins by tryptic peptide mass fingerprinting (done by the Servicio de Proteomica, CIC, Salamanca, Spain) was performed as described previously (24).

Computational methods. Similarity searches were performed using BLAST software (1) at the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov). Protein domains or motifs were searched for in Abz2 using the InterProScan program and the InterPro database of protein families at the European Bioinformatics Institute (www.ebi.ac.uk). Multiple sequence alignments were done with CLUSTAL W (41), using the European Bioinformatics Institute tools (www.ebi.ac.uk). Molecular phylogenetic analysis was carried out by using MEGA v 3.1 software (22).

RESULTS

Genome-wide screening for sulfanilamide-hypersensitive mutants. Using the collection of yeast haploid knockout strains and the heterozygous essential diploids set, we performed a chemical genomic screening to identify the spectrum of genes whose deletion alters the fitness profile in the presence of the PABA antimetabolite sulfanilamide. Although the majority of the deletion strains grown grew comparably to wild-type cells on SMM supplemented with sulfanilamide (200 μg/ml), a large number of mutant strains showed mild growth retardation (data not shown). At least 117 strains, however, showed a severe growth defect (75% GI) caused by the presence of the antifolate drug in the medium. Functional classification of the deletion strains grown grew comparably to wild-type cells whose deletion alters the fitness profile in the presence of the PABA antimetabolite sulfanilamide. Although the majority of the deletion of the uncharacterized previously known member of the PABA biosynthetic pathway, folic acid, thus demonstrating that no mutation other than the deletion of the ABZ2 (YMR289w) gene was responsible for the auxotrophy of the mutant.

Identification of YMR289W as the ABZ2 gene. In the present study we focused on YMR289w. Further characterization of the ymr289w mutant revealed a slow growth on SMM, a phenotype that resulted in the inability to grow after two successive subcultures in the same medium. This suggests that the ymr289w mutant strain is impaired in the synthesis of an essential metabolite whose cellular pool is exhausted after prolonged culture. The hypersensitivity to the antifolate sulfanilamide displayed by ymr289w cells points to the folic acid biosynthetic pathway as being responsible for the auxotrophy of the mutant. This hypothesis was confirmed by the fact that the addition of folic acid (2 μg/liter) to the minimal medium restored the ability of the mutant to grow (Fig. 2). Supplementation of the minimal medium with PABA (200 ng/ml) also rescued the growth defect of the mutant cells, indicating that the YMR289w gene (named ABZ2 according to the yeast gene nomenclature guidelines) is involved in the biosynthesis of this folic acid precursor. A YEp352-derived multicopy plasmid harboring the cognate YMR289w gene also rendered the ymr289w (abz2) mutant able to grow in a minimal medium lacking PABA or folic acid, thus demonstrating that no mutation other than the deletion of the ABZ2 (YMR289w) gene was responsible for the PABA auxotrophy.

ABZ2 functionally complements an E. coli pabC mutant. The synthesis of PABA in E. coli is carried out by three enzymes encoded by the genes designated pabA, pabB, and pabC. In yeast, an ADC synthase gene (ABZ1) has been described that encodes a bifunctional protein containing the two domains homologous to the two components—PabA and PabB—of the prokaryotic ADC synthase (9). The identification in S. cerevisiae of a second gene, ABZ2, involved in the biosynthesis
PABA suggests that this gene is functionally homologous to the bacterial pabC gene.

To check that ABZ2 was able to complement the deficiency of the pabC function, we constructed an E. coli pabC single-gene mutant by the PCR-based inactivation method as described in Materials and Methods (8). One ΔpabC mutant, designated JR3125, was verified by PCR amplification and sequencing and was selected for the complementation assays.

The deletion of the pabC gene in the JR3125 strain resulted in a severe impairment of growth in M9 minimal medium that could be rescued by the addition of PABA (200 ng/ml) to the medium (Fig. 3). The transformation of JR3125 with a YEp352-derived vector containing the complete ABZ2 ORF and 329 and 441 bp of the upstream and downstream regions, respectively, was also able to restore the ability to grow in the absence of PABA up to nearly the wild-type rate of growth (Fig. 3). However, no complementation was seen in the pabC deletion mutant transformed with the empty vector. These results show that the ABZ2 gene is the yeast functional homologue of the prokaryotic pabC gene.

Recombinant Abz2p has ADC lyase activity. The complementation assay and the sequence analyses suggested that ABZ2 encodes an ADC lyase in yeast. A direct test of the hypothesis that Abz2p catalyzes the β-elimination of pyruvate and aromatization of the ADC to give PABA required the preparation of the pure polypeptide. Since Abz2p is a scarce protein in yeast (11), the Abz2 polypeptide was overexpressed in E. coli as a fusion protein containing an N-terminal His6 tag. The N-terminal extension did not interfere with activity, since the expression of this fusion protein in the E. coli ΔpabC mutant restored growth on M9 medium lacking PABA and resulted in a 50-fold increase in ADC lyase activity in E. coli whole-cell extracts (not shown). The Abz2p recombinant protein was purified as described in Materials and Methods and used in vitro ADC lyase assays. Because the 4-amino-4-deoxychorismate substrate was not commercially available, we prepared it enzymatically from chorismate and glutamine using recombinant ADC synthase from yeast in a coupled assay. ADC synthase, the product of ABZ1, catalyzes the synthesis of 4-amino-4-deoxychorismate by replacing the hydroxyl group of chorismate with an amino group acid supplied by glutamine (9).

The substrate chorismate, the intermediate ADC, and the final product PABA all absorb at 280 nm but can be readily separated by reversed-phase high-pressure liquid chromatography (HPLC). We confirmed this chromatographic separation with elution times comparable to that reported previously (14). Therefore, after HPLC separation it is possible to determine the conversion of chorismate to ADC due to the enzymatic action of Abz1p; subsequent addition of ADC lyase to the reaction mixture can be used to analyze the conversion of the ADC intermediate to PABA. Addition to the reaction mixture of purified Abz1p led to a decrease in chorismate and the concomitant formation of ADC. After 2 h of incubation almost a total conversion of chorismate into ADC was achieved (Fig. 4). At this time, purified Abz2p protein was added to the reaction
mixture. The presence of Abz2p catalyzed the conversion of ADC into PABA in a time-dependent manner. Control reactions revealed that ADC synthase and ADC lyase enzymatic activities were dependent on the presence of Abz1 and Abz2 proteins, respectively (data not shown). Thus, these results show that ABZ2 encodes a functional ADC lyase that catalyzes the last step in the synthesis of PABA of the folic acid pathway.

**Dimeric structure of yeast ADC lyase.** Most class IV amino acid aminotransferase-like pyridoxal phosphate-dependent enzymes are homodimers (10). Moreover, the crystal structure of E. coli ADC lyase has been recently determined and shown to form homodimers (29). The subunit composition of Abz2p was analyzed by using blue native gel electrophoresis. Apparently pure recombinant Abz2p was applied to a 4 to 16% gradient blue native gel (Fig. 5A). Two dominant forms could be identified with approximate molecular masses of 50 and 100 kDa. The estimated molecular mass of the smaller form corresponded well to that of the monomeric Abz2 polypeptide, as calculated from its amino acid sequence and demonstrated by SDS-PAGE electrophoresis analysis (45 kDa) (Fig. 5A). The larger and slightly less abundant one apparently represents the Abz2p homodimer. The identity of both complexes was confirmed by tryptic peptide mass fingerprinting (data not shown).

The dimeric state of Abz2p was also investigated by analytical size exclusion chromatography on a Superdex 200 column calibrated with soluble proteins. The recombinant Abz2p preparation eluted in two distinct peaks, with apparent molecular masses of 45 and 92 kDa, in agreement with the sizes expected for the monomeric and homodimeric forms of Abz2p, respectively (Fig. 5B). ADC lyase activity analyses of the eluted fractions revealed that only fractions containing the dimeric Abz2p were enzymatically active, indicating that dimerization is indispensable for catalytic activity (Fig. 5B).

**Fungal ADC lyases are distantly related to prokaryotic and plant ADC lyases.** A BLASTp search of the whole GenBank database with the deduced protein product of ABZ2 detected as the closest homologues a group of proteins of unknown function belonging to different fungal species. These proteins show sufficient homology to Abz2p (identity percentages ranging from 37% for Ashbya gossypii to 26% for Neurospora crassa) to predict that they represent authentic ADC lyases in those fungal species (Fig. 6A). Strikingly, specialized BLAST searches with Abz2p restricted to the bacterial or plant entries of the GenBank database failed to detect homologues, although experimentally characterized ADC lyases have been described in E. coli, tomato, and Arabidopsis thaliana. Phylogenetic analysis of representative ADC lyase sequences confirmed that the fungal ADC lyase group clustered well apart from the bacterial and plant ADC lyase groups, although clearly belonging to an ADC lyase subfamily of proteins that is divergent from the structurally related branched-chain amino acid aminotransferase family (Fig. 6B). In fact, a search of the InterPro database (36) for domains or protein motifs present in Abz2p revealed the presence of a domain characteristic of the amino acid aminotransferase-like pyridoxal phosphate-dependent enzymes. In addition to ADC lyases, this superfamily of proteins also encompasses branched-chain amino acid aminotransferases and prokaryotic D-aminotransferases, which are characterized by the presence of a pyridoxal phosphate-binding site located at the interface of a large and a small domain linked by a flexible loop (29). Important residues that are believed to be catalytically essential in ADC lyases are all conserved in Abz2p, as well as in the fungal homologues analyzed in the present study (Fig. 6A).

**DISCUSSION**

Despite the overwhelming impact of systematic genome sequencing on the understanding of the biology and evolution of organisms, enormous gaps remain in our current knowledge of
and dihydrofolate (34), therefore reducing the pool of folate cofactors and affecting a variety of biosynthetic pathways dependent on one-carbon transfer, including the synthesis of purines, thymidylate, N-formylmethionyl-tRNA, and some amino acids. Indeed, the screening identified a broad range of gene functions affected by sulfanilamide-induced cytotoxicity (Table 1). As expected, we found a significant number of mutants involved directly or indirectly in one-carbon/folate metabolism (abz1, aro1, and fol2), sulfur metabolism (ckb1, ckb2, gsh1, hrt1, met6, met18, met28, and met30), and nucleotide metabolism (ade6, adk1, cdc8, guk1, pho2, rib3, mr2, and trn7), highlighting the validity of our experimental approach.

Folate pool depletion indirectly blocks dTMP production, leading to dTTP depletion, misincorporation of uracil into DNA during replication, and imbalance in deoxynucleoside triphosphate pools, ultimately causing DNA damage and cytotoxicity due to the so-called “thymineless death” (23, 25). Interestingly, the screening reproducibly yielded mutants lacking deoxynucleoside monophosphate kinase activities (adk1, cdc8, and guk1) or defective in other regulatory or enzymatic functions of nucleotide metabolism (ade6, pho2, and mr2). This presumably reflects the detrimental effects of the nucleotide imbalance in the presence of the folate depletant agent. Moreover, DNA repair and DNA replication mutants (fyv6, ino80, rad52, rfc2, and rrn3, and DNA polymerase δ subunits cdc2, hys2, and pol32), as well as mutants involved in chromatin...
A

B
modification (arp5, arp8, gcn5, rsc6, rtt109, sgf20, snf2, spc3, sib1, and swd1), are also severely compromised by the sulfa drug, as expected from the damaging cycles of uracil DNA misincorporation and attempted DNA repair of deoxyuridine residues.

We presume that the sulfonamide hypersensitivity of some mutants involved in cell homeostasis and vesicular transport is likely to be unspecific, since these mutants are affected in processes related to drug or stress responses and show pleiotropic defects in the presence of different insults (32, 45).

Several unexpected mutants involved in lipid metabolism and its regulation were also susceptible to sulfanilamide treatment. Of particular interest are the mutants affected in ergosterol biosynthesis, which—besides its role in membrane fluidity and permeability—have been linked to folate metabolism via δ-adenosyl-l-methionine (31).

All of these observations reflect the complex phenotypes caused by sulfanilamide-mediated folate depletion, due directly to a one-carbon unit deficiency and indirectly affecting other processes, since many methylated molecules such as nucleic acids, proteins, and lipids are formed by transmethylation reactions with δ-adenosyl-l-methionine. It is noteworthy that our findings could open new fields for exploring novel treatments for several bacterial, fungal, and protozoan infections because the combination of sulfa drugs with inhibitors of some of the sulfanilamide-compromised processes found could render these pathogens sensitive to combined therapy. Further research will be required to assess precisely how the genes identified influence the response to sulfanilamide treatment.

Among the genes whose deletion elicits sulfanilamide hypersensitivity, YHI9 and YMR289w lack specific physiological functions. The three-dimensional crystal structure of Yhi9p has recently been determined, and it is classified as a member of the PhzF (PF02567) enzyme family that might use either chorismate or an anthranilate derivative as a substrate (27). No of the PhzF (PF02567) enzyme family that might use either chorismate or an anthranilate derivative as a substrate (27). No functional or structural data on YMR289w were available prior to this work.

Here we have demonstrated that the YMR289w ORF (ABZ2 gene) of S. cerevisiae encodes an enzyme responsible for the last of the two steps in the PABA branch of the folate biosynthetic pathway. Heterologous complementation of an E. coli pabC mutation and direct enzymatic characterization of the Abz2 protein support this notion. Yeast abz2 mutants impaired in the synthesis of PABA are able to grow on minimal medium supplemented either with PABA or with folic acid, indicating that this metabolite does not have an essential function other than its role as a substrate in the synthesis of folates.

Yeast mutants deleted in ABZ2 appear to be leaky for PABA auxotrophy. This characteristic, also observed in E. coli pabC mutants (13), has tacitly been attributed to the fact that ADC is unstable and is spontaneously converted to PABA in a nonenzymatic form (40). However, the apparent leakiness of the abz2 mutants seems to be due to the existence in the cells of a PABA or a folate pool sufficient to support the growth and division of the cells for several generations. As described here, subculturing of the abz2 mutant in medium lacking PABA or folate exacerbates its PABA auxotrophy, presumably because of the depletion of existing PABA or folate pools. In plants, most of the endogenous PABA content is not present as free acid but as a glucose ester (p-amino benzoyl-β-d-glucopyranoside [PABA-Glc]). This conjugated PABA-Glc form is accumulated in the cytoplasm and has been proposed to serve as an accessible storage depot of PABA for use as a substrate to sustain the biosynthesis of folate that takes place in mitochondria (37). Although the formation of PABA-Glc has been not detected in yeast (37), it is likely that an equivalent storage form of PABA necessary to drive its transport to mitochondria would exist in this organism.

Although the analysis of S. cerevisiae ADC lyase revealed that the protein has no significant sequence homology to its bacterial or plant counterparts, the yeast enzyme does contain a domain typical of the class IV amino acid aminotransferases family (36). Furthermore, all of the residues proposed to be catalytically essential in E. coli ADC lyase (29) are also present in Abz2p (Fig. 6A). Thus, the conserved lysine residue that covalently binds pyridoxal phosphate can be found in position 251 in the yeast enzyme. Also present are residues Arg107 and Glu297, which may directly interact with the enzyme and play a critical role in the catalytic function. Finally, the carboxylate groups of ADC could be recognized by Asn360 and Arg182, and the cyclohexadiene moiety could make van der Waals contact with the side chain of Leu550 (Fig. 6A).

Yeast ADC lyase appears to form dimers, a structural characteristic that is also shared by the E. coli and A. thaliana ADC lyases (5, 13, 29). Whereas the prokaryotic and plant enzymes have been detected only in their dimeric form, our analyses using blue native electrophoresis and size exclusion chromatography indicate that the dimeric form of the enzyme is in equilibrium with the monomeric form, although only the dimers are catalytically active. This equilibrium between active

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**FIG. 6.** Comparison of S. cerevisiae ADC lyase to other fungal homologues and the experimentally characterized E. coli and A. thaliana ADC lyases. (A) Alignment of the deduced protein sequences from S. cerevisiae (S.c.; NP_014016), A. gossypii (A.g.; NP_986096), Candida albicans (C.a.; XP_715312), Yarrowia lipolytica (Y.l.; XP_502983), Schizosaccharomyces pombe (S.p.; NP_595968), E. coli (E.c.; A42954), and A. thaliana (A.t.; NP_200593). Conserved residues believed to be catalytically important according to the structure of E. coli ADC lyase (29) are boxed. (B) Molecular phylogenetic tree of inferred ADC lyase (ADCL) and structurally related branched-chain amino acid transaminase (BCAT) protein sequences from fungi, plants, and bacteria. The circled zone demarcates the fungal sequences. ADCL: Sc, S. cerevisiae; Ag, A. gossypii; Ca, C. albicans; Sp, S. pombe; K1, Kluyveromyces lactis (XP_451419); Nc, Neurospora crassa (XP_961237); Ec, E. coli; Bs, Bacillus subtilis (NP_387957); At, A. thaliana; Lc, Lycopersicon esculentum (AY547289); BCAT1-Sc (NP_012078); BCAT2-Sc (NP_012682); BCAT_Ec (P00510); BCAT1_At (AAC34335); BCAT2_At (AAC34333); BCAT3_At (CACB6936); BACT5-At (BAB10685); BACT6-At (AA76437). The phylogenetic tree was constructed by using the neighbor-joining method of MEGA 3.1 (22). Each node was tested by using the bootstrap approach by taking 1,000 replications and a random seeding of 64,238 to ascertain the reliability of the nodes. The numbers indicated in parentheses are in percentages against each node. Branch lengths were measured in terms of amino acid substitutions, with the scale indicated below the tree.
dimers and inactive monomers could be the basis of a mechanism of activity regulation of this enzyme, which does not appear to be feedback regulated for PABA or folates (5, 42), and neither does its expression seem to be subject to transcriptional control (our unpublished results). Although unlikely, the possibility still exists that the presence of Abz2p monomers in the enzyme preparation could be an artifact due to the presence of an N-terminal His6 tag in the recombinant protein used in our analyses.

In contrast to plants, green fluorescent protein-tagged Abz2p is clearly located in the cytoplasm (results not shown), a finding in agreement with a previous report (21). Since Abz1p is also a cytoplasmic enzyme, it is clear that the synthesis of PABA in S. cerevisiae takes place in the cytoplasm and not inside a specific organelle, as occurs in the plastids of plants. In this respect, the presence of PABA is freely distributed in the cytoplasm of fungi raises the issue of the possible existence of PABA in the cytoplasm of fungi. In this respect, the presence of PABA freely distributed in the cytoplasm of fungi raises the issue of the possible existence of an N-terminal His6 tag in the recombinant protein used in our analyses.

The identification in the present study of the last unknown gene involved in the biosynthesis of tetrahydrofolate in yeast enables the initiation of metabolic engineering projects aimed to the construction of valuable vitamin B9 producer strains. In particular, overexpression of the PABA biosynthetic pathway could solve one of the major constraints of folate synthesis, namely, the supply of the PABA precursor.

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