Nucleosides, Nucleotides, and Nucleic Acids, 27:794–799, 2008

ISSN: 1525-7770 print / 1532-2335 online
DOI: 10.1080/15257770802145983

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DEGRADATION OF PYRIMIDINES IN *SACCHAROMYCES KLUYVERI*: TRANSAMINATION OF β-ALANINE

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□ *Beta-alanine is an intermediate in the reductive degradation of uracil. Recently we have identified and characterized the* Saccharomyces kluyveri PYD4 *gene and the corresponding enzyme β-alanine aminotransferase (^{Sk}Pyd4p), highly homologous to eukaryotic γ-aminobutyrate aminotransferase (GABA-AT).* S. kluyveri *has two aminotransferases, GABA aminotransferase (^{Sk}Uga1p) with 80% and ^{Sk}Pyd4p with 55% identity to* S. cerevisiae *GABA-AT. ^{Sk}Pyd4p is a typical pyridoxal phosphate-dependent aminotransferase, specific for α-ketoglutarate (αKG), β-alanine (BAL) and γ-aminobutyrate (GABA), showing a ping-pong kinetic mechanism involving two half-reactions and substrate inhibition. ^{Sk}Uga1p accepts only αKG and GABA but not BAL, thus only ^{Sk}Pydy4p belongs to the uracil degradative pathway.*

Keywords Aminotransferase; β-alanine; γ-aminobutyrate; gene duplication; *Saccharomyces kluyveri*

INTRODUCTION

In biological systems, β-alanine (BAL) plays a major role as a precursor of pantothenic acid,[1] a constituent of dipeptides such as anserine and carnosine in the brain[2] and as an intermediate in the catabolic pathway of pyrimidines. β-Alanine is a structural analog of γ-aminobutyrate (GABA) and glycine, two major inhibitory neurotransmitters.[3] Moreover, BAL itself has been suggested to be involved in synaptic transmission. Recent studies revealed that BAL activates the same receptors on the membranes of rat sacral dorsal commissural neurons as glyine.[4] In mammalian systems, uracil is degraded to BAL in three consecutive enzymatic steps by dihydropyrimidine dehydrogenase, dihydropyrimidine amidohydrolase and β-alanine synthase. In contrast, microorganisms produce BAL mostly by direct

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decarboxylation of L-aspartate.[5] Bacteria and plants can also degrade spermidine to BAL.[6]

Mammals degrade BAL to malonate semialdehyde (MSA) using either BAL:α-ketoglutarate aminotransferase (BAL-AT) or D-3-aminoisobutyrate: pyruvate aminotransferase (DAIB-AT). The produced MSA is subsequently metabolized by semialdehyde dehydrogenase to acetyl-CoA.[7] In humans, BAL-AT and GABA aminotransferase activity resides within a single enzyme.[8]

Even though *Saccharomyces cerevisae* can synthesize BAL from polyamines, it cannot use BAL as sole nitrogen source.[9] GABA, in contrast, is readily used as sole nitrogen source by the action of GABA-AT and succinic semialdehyde dehydrogenase (SSADH), encoded by *UGA1* and *UGA2*, respectively.[10] Several yeast species, like *Saccharomyces kluyveri* and *Schizosaccharomyces pombe* can utilize BAL as sole nitrogen source.[11]

In this study, we present the identification and characterization of two *S. kluyveri* enzymes involved in transamination of BAL and GABA, encoded by the *PYD4* and *UGA1* genes.

MATERIALS AND METHODS

All chemicals and enzymes were purchased from Sigma (St. Louis, MO, USA). Yeast nitrogen base without amino acids and ammonium sulfate were obtained from Difco (BD Biosciences, San Jose, CA, USA) and chelating Sepharose fast flow from Amersham Biosciences (Piscataway, NJ, USA). Oligos were purchased from DNA Technology (Aarhus, Denmark).

Strains and Media

The yeast and bacterial strains used in this study were grown in YPD medium (1% yeast extract, 2% bactopeptone, 2% glucose) or synthetic defined medium (1% succinic acid, 0.6% sodium hydroxide, 2% glucose, 0.67% yeast nitrogen base without amino acids and ammonium sulfate) supplemented with different nitrogen sources (0.5% ammonium sulfate, 0.1% for all other types). For solid medium (plates) 2% agar was added. For plasmid rescue, *E. coli* strain XL1-Blue was used, and for cloning TOP10 and for overexpression of fusion proteins, BL21 Star. All bacteria were grown at 37°C in LB medium supplemented with ampicillin (100 mg $\times$ L^{-1}).

Mutagenesis

S. kluyveri mutants were generated from strains Y156 and 159 with ethyl methanesulfonate.[11] Mutagenized cells were plated and grown for 2–3 days at 25°C. The plates were replicated onto new plates containing ammonium sulfate (control) or dihydrouracil (DHU) as sole nitrogen

source. After 5–7 days at 25°C, colonies were selected based on their inability to grow on DHU plates. Putative mutants were tested for growth on β-ureidopropionate (BUP), with BAL as sole nitrogen source.

Cloning, Expression, and Purification of SkPyd4p

The cloning, expression and purification are described in detail elsewhere.[12]

Clones of succinic semialdehyde dehydrogenase (SSADH) and malonate semialdehyde decarboxylase (MSADC) were kindly provided by M. D. Toney (University of California at Davis) and C. P. Whitman (University of Texas, Austin) and the proteins obtained as previously described.[13,14]

Spectrophotometric Measurements

All measurements were performed on a Varian Cary 3 UV/Visible spectrophotometer equipped with a temperature controlled automatic multicell changer.

Enzyme Assays

In a continuous assay, the production of MSA by BAL–AT is coupled with MSADC to produce acetaldehyde, which in a NADH-dependent alcohol dehydrogenase reaction generates NAD^+ and ethanol. The MSADC-generated acetaldehyde was monitored by observing the NADH depletion at 340 nm.[12] In a continuous assay, succinic semialdehyde (SSA) produced by Uga1p and Pyd4p was converted by the NADP+-dependent SSADH to NADPH and succinate. The absorbance change at 340 nm was monitored.[12]

Data Analysis

Kinetic data analysis was performed as described previously.[12]

RESULTS

Identification of the *PYD4* Gene Encoding a β-Alanine Aminotransferase

While screening mutagenized *S. kluyveri* cells grown on DHU, a mutant, Y947, was isolated which could not use DHU, BUP (β-ureidopropionate) or BAL as sole nitrogen source. This mutant behaved like a double mutant, since both BUP and BAL can serve as a direct nitrogen source via the action of β-alanine synthase and BAL-AT. However, complementation with the genomic library yielded plasmid P733, which supported growth on all three

nitrogen sources (DHU, BUP, and BAL). An open reading frame, termed *PYD4* (DQ512721), encoding a protein with 55% identity at the protein level to *S. cerevisiae UGA1* gene product, GABA-AT, was identified. A BLAST search of ^{Sc}*UGA1* in the *S. kluyveri* genome revealed another homologous gene, termed ^{Sk}*UGA1* (DQ512722). Its gene product has 80% identity to ^{Sc}Uga1p, and 57% to ^{Sk}Pyd4p.

Sequence Analysis of the *PYD4* Gene

Phylogenetic analysis of the homologous proteins from yeast shows that *PYD4* is only found in two other sequenced yeasts (*Debaryomyces hansenii* and *Candida albicans*). Both yeasts also contain a putative *UGA1* gene. A search for β-alanine synthase (encoded by *PYD3*) and dihydropyrimidine amidohydrolase (encoded by *PYD2*) revealed that *S. kluyveri* is the only yeast carrying all three *PYD* genes. *C. albicans* and *D. hansenii* lack *PYD2*, and *Kluyveromyces lactis* has *PYD2* and *PYD3*, but lacks *PYD4*. *S. cerevisiae* lacks *PYD2*, *PYD3,* and *PYD4*.

Purification and Properies of ^{Sk}Pyd4p and ^{Sk}Uga1p

The purification of the His-tagged ^{Sk}Pyd4p and ^{Sk}Uga1p was accomplished after disruption of the respective cells in three steps using streptomycin treatment, affinity chromatography on nickel-chelating Sepharose and size exclusion gel filtration on Sepharose S-12. From 1 liter of cell culture about 20 mg of pure protein could be obtained. The molecular weight of the subunits of both proteins was found to be about 56-57 kDa based on SDS/PAGE gel. Purified ^{Sk}Pyd4p and ^{Sk}Uga1p had native molecular masses of 111 (dimers) and 135/240 kDa (dimers/tetramers), respectively. The spectra of purified ^{Sk}Pyd4p and ^{Sk}Uga1p show two maxima at 340 and 410 nm, indicating the equilibrium of the ketoenamine (410 nm) and enolimine (340 nm) tautomers which can be shifted by changing the pH of the protein solution.

Initial Velocity Studies

In the initial reaction of ^{Sk}Pyd4p, αKG and BAL (or GABA) are converted to glutamate and MSA (or SSA), respectively. In a second reaction, either the amount of MSA or SSA was determined. The substrate specificity of ^{Sk}Pyd4p was tested and it could be shown that ^{Sk}Pyd4p accepts both BAL and GABA as amino donors but with a preference for BAL. In contrast, ^{Sk}Uga1p uses only GABA as substrate not BAL.

Both ^{Sk}Pyd4p and ^{Sk}Uga1p were subjected to a full kinetic analysis. Since ^{Sk}Uga1p shows no activity towards BAL, only ^{Sk}Pyd4p was analyzed with both substrates. When the initial velocity was determined at various αKG

concentrations and several fixed levels of BAL, a pattern composed of parallel lines is seen at low reactant concentrations in a double reciprocal plot. At high αKG concentrations, the linear lines are deflected upwards, indicating substrate inhibition. Values of $K_{M,BAL}$, $K_{M,GABA}$ and $K_{M,\alpha KG}$ with 8.2 ± 0.4, 1.8 ± 0.2, 2.9 ± 0.2 mM, respectively, were determined for ^{Sk}Pyd4p. Since the maximal velocity for ^{Sk}Pyd4p (7.5 units/mg) is more than eight times higher for BAL, ^{Sk}Pyd4p can be considered a BAL-AT rather than a GABA-AT. ^{Sk}Uga1p exhibits $K_{M,GABA}$and $K_{M,\alpha KG}$ values of 3.2 ± 0.2 and 0.22 ± 0.01 mM, respectively. The obtained kinetic behaviour is consistent with a ping-pong kinetic mechanism.

DISCUSSION

Mammals have only one gene encoding a GABA-AT which exhibits both BAL-AT and GABA-AT activity. *S. cerevisiae* has a single gene encoding GABA-AT (*^{Sc}UGA1*), while *S. kluyveri* has two *^{Sc}UGA1*-like genes. Similarly, the genomes of *C. albicans* and *D. hansenii* each contain an *UGA1*-like gene. This study identifies that one of the two genes in *S. kluyveri*, *PYD4*, encodes a BAL-AT involved in BAL degradation. Apparently, the original *PYD4/UGA1* gene was duplicated in one yeast lineage. Later on, one of the duplicated genes was lost in some descendant lineages, such as *S. cerevisiae*. The time point for the gene duplication is estimated to be ~200 million years ago, when *Yarrowia lipolytic*a split from the *Saccharomyces/Candida/Debaryomyces* lineages.[15]

The pattern of an array of parallel lines for different fixed concentrations of the second substrate is consistent with a ping-pong kinetic mechanism. All aminotransferases adhere to such a mechanism, where the keto substrates exert substrate inhibition. Both ^{Sk}Pyd4p and GABA-AT from *E. coli* show comparable substrate inhibition for αKG with inhibition constants of 28.3 ± 6.7 and 10.2 ± 2.3 mM,[14] respectively. Both *Sk*Pyd4p and *Sk*Uga1p show a reaction mechanism involving two half-reactions.

Our study shows that *PYD4/*^{Sk}Pyd4p is a part of the reductive pathway of pyrimidine nucleic acid precursors (Figure 1).

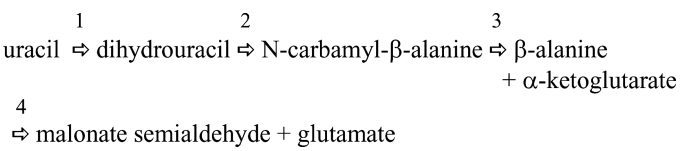

FIGURE 1 Reductive degradation of pyrimidine nucleic acid percursors. Uracil is degraded via dihydrouracil, N-carbamyl-*β*-alanine, *β*-alanine to malonate semialdehyde by dihydropyrimidine dehydrogenase (1, encoded by *PYD1*), dihydropyrimidine amidohydrolase (2, encoded by *PYD2*), *β*-alanine synthase (3, encoded by *PYD3*), and *β*-alanine aminotransferase (4, encoded by *PYD4*), respectively.[16]

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