Pyridoxine biosynthesis in yeast: participation of ribose 5-phosphate ketol-isomerase

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To identify the genes involved in pyridoxine synthesis in yeast, auxotrophic mutants were prepared. After transformation with a yeast genomic library, a transformant (A22t1) was obtained from one of the auxotrophs, A22, which lost the pyridoxine auxotrophy. From an analysis of the plasmid harboured in A22t1, the *RKII* gene coding for ribose 5-phosphate ketol-isomerase and residing on chromosome no. 15 was identified as the responsible gene. This notion was confirmed by gene disruption and tetrad analysis on a diploid prepared from the wild-type and the auxotroph. The site of mutation on the *RKII* gene was identified as position 566 with a transition from guanine to adenine, resulting in amino

acid substitution of Arg-189 with lysine. The enzymic activity of the Arg¹⁸⁹ → Lys (R189K) mutant of ribose 5-phosphate ketol-isomerase was 0.6 % when compared with the wild-type enzyme. Loss of the structural integrity of the protein seems to be responsible for the greatly diminished activity, which eventually leads to a shortage of either ribose 5-phosphate or ribulose 5-phosphate as the starting or intermediary material for pyridoxine synthesis.

Key words: pyridoxine, ribose 5-phosphate ketol-isomerase, ribulose 5-phosphate, *RKII*, vitamin B₆.

INTRODUCTION

The coenzyme pyridoxal phosphate plays an important role in amino acid metabolism. Pyridoxine is the precursor to pyridoxal phosphate and the major form of vitamin B₆. Two pathways are known for the biosynthesis of pyridoxine. The first, found in *Escherichia coli* and some other prokaryotes, has been studied extensively over the last several decades and now is fairly well understood [1–3]. In essence, the synthesis begins with erythrose 4-phosphate and 1-deoxy-D-xylulose 5-phosphate. After several chemical transformations of the former, the end-product, 1-amino-3-phosphohydroxypropane-2-one, undergoes condensation with the latter in the last step of the synthesis to give pyridoxine 5'-phosphate. The other route, found in plants, fungi and some eubacteria such as *Bacillus subtilis*, has just started to be clarified. The origin of the ring nitrogen of pyridoxine is different between the two pathways: the ring nitrogen originates from the amide group of glutamine in *Saccharomyces cerevisiae* [4], whereas the α -amino group of glutamate is the source of the nitrogen of pyridoxine in *E. coli* [5]. Previously, two independent groups identified *pyroA* and *SOR1* (*PDX1*) as the genes responsible for pyridoxine synthesis in *Aspergillus nidulans* and *Cercospora nicotianae* respectively [6,7]. They are homologous genes and their homologues are distributed widely in various organisms such as yeast in *SNZ1*, *SNZ2* and *SNZ3*. In addition, *PDX2* was also identified recently [8], and its homologues are also distributed in yeast as *SNO1*, *SNO2* and *SNO3*. Recently, we observed that the complex of Snz1p and Sno1p serves as a glutaminase with the latter hydrolysing glutamine. It was also reported recently that a pentose or pentulose constitutes the skeleton of pyridoxine in yeast and the initial product of synthesis is 2'-hydroxypyridoxine [9,10]. In the present study, we report that ribose 5-phosphate ketol-isomerase, the translation product of the *RKII* gene, which mediates the interconversion of ribose 5-phosphate and ribulose 5-phosphate, plays a crucial role in pyridoxine synthesis in yeast.

MATERIALS AND METHODS

Materials

Inorganic salts and common organic chemicals including amino acids, nucleic bases and vitamins were obtained from local commercial sources. D-ribose 5-phosphate and D-fructose 5-phosphate were from Sigma (St. Louis, MO, U.S.A.). Reagents for genetic engineering, such as restriction enzymes, were purchased from Takara (Kyoto, Japan) and New England Biolabs (Beverly, MA, U.S.A.). Oligonucleotides were custom-synthesized by Hokkaido Science (Sapporo, Japan).

Strains and media

Yeast strains used in the present study and their genotypes are shown in Table 1. The following media were used to grow yeast: YPD [1 % (w/v) yeast extract/2 % (w/v) peptone/2 % (w/v) glucose] and synthetic medium [20 g of glucose, 1.02 g of (NH₄)₂SO₄, 0.875 g of KH₂PO₄, 0.125 g of K₂HPO₄, 0.02 g of CaCl₂ · H₂O, 0.01 g of NaCl, 0.05 g of MgSO₄ · 7H₂O, 40 μ g of CuSO₄ · 5H₂O, 400 μ g of MnSO₄ · H₂O, 200 μ g of FeCl₃ · 6H₂O, 400 μ g of ZnSO₄ · 7H₂O, 200 μ g of Na₂MoO₄ · 2H₂O, 100 μ g of KI, 500 μ g of H₃BO₃, 2 μ g of biotin, 10 mg of inositol, 0.2 mg of nicotinic acid and 0.2 mg/l of calcium pantothenate]. Where indicated, histidine, leucine, tryptophan, uracil and pyridoxine were supplemented to the synthetic media at a final concentration of 20 mg/l. The medium used for sporulation contained 10 g of potassium acetate, 1 g of yeast extract and 0.5 g/l of glucose. Solid media contained 2 % agar. For each liquid culture experiment, yeast cells were shaken at 250 rev./min in 10 ml of medium at 30 °C for the time specified.

General procedures

All of the gene manipulations of *S. cerevisiae* and *E. coli* were performed by standard methods [11,12]. PCR was run on an Astec

Abbreviations used: RPE, ribulose 5-phosphate epimerase; TKL, transketolase.

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Table 1 *S. cerevisiae* strains used in the present study

Strain	Genotype
D451-3	MAT α , <i>leu2</i> , <i>ura3</i>
D452-2	MAT α , <i>his3</i> , <i>leu2</i> , <i>ura3</i>
D373-1	MAT α , <i>his3</i> , <i>leu2</i> , <i>trp1</i>
A22	Pyridoxine auxotroph from D451-3
A22t1	A22t + pYN1
A22t2	A22t + pYN2
A22t3	A22t + pYN3
A22t4	A22t + pYN4
Da4	MAT α/α , <i>HIS3/his3</i> , <i>leu2/leu2</i> , <i>ura3/ura3</i>

PC-700 (Astec, Tokyo, Japan). Sequencing of plasmids was performed by the dideoxy chain-termination method on an automated DNA sequencer model DSQ1000 (Shimadzu, Kyoto, Japan). Yeast genomic DNA sequences were retrieved from the database site (<http://genome-www.stanford.edu/Saccharomyces/>). Protein sequences were determined on an Applied Biosystems 491A sequencer. CD spectra were determined on a Jasco J725 spectrophotometer at an ambient temperature. HPLC was run on a Hitachi chromatograph with a Tosoh TSK gel SW3000 as the stationary phase. Shuttle vectors YEpM4 (multiple copy) and YCpL2 (single copy) were used for the introduction of genes into yeast cells [13,14].

Preparation of pyridoxine auxotrophs

Pyridoxine auxotrophic mutants were produced by ethyl methane-sulphonate mutagenesis [15]. In brief, cells were treated with ethyl methanesulphonate for 50 min and then spread over the entire surface of synthetic medium plates supplemented with pyridoxine and growth requirements, and the plates were incubated at 30 °C. The colonies that appeared on the supplemented plates were then transferred by replica plating first to a minimal plate (–PN) and then to one supplemented with pyridoxine (+PN). The plates were scored for colonies, which appeared in +PN media but not in the –PN media. This selection process was repeated several times to ensure that the colonies unable to grow in –PN medium are indeed pyridoxine auxotrophs.

Transformation of yeast cells

Pyridoxine auxotrophic mutants were transformed with a YEpM4-based genomic library [13] or subcloned plasmids for complementation and with a gene-disrupted fragment for gene disruption by the lithium acetate method [16]. Transformants were selected on synthetic medium plates containing appropriate supplements.

Subcloning of pYN1

Plasmid pYN1, harbouring a 4.1 kb fragment of chromosome no. 15, was digested with restriction endonucleases *Bsp*EI and *Hind*III to remove most of the *ARF3* (Figure 1). The remaining part of pYN1 was purified with the GeneClean II kit (BIO 101, La Jolla, CA, U.S.A.), then subjected to Klenow fragment treatment and self-ligated to give plasmid pYN2 carrying *RK11* only. The *Sal*I–*Pst*I fragment (2.6 kb) having the *ARF3* was excised from pYN1 and subcloned into vector YEpM4 to give pYN3.

For subcloning of *RK11* into a single-copy vector YCpL2, the *Bsp*EI–*Nhe*I fragment (2.2 kb) was excised from the original

Figure 1 (A) Partial map of *S. cerevisiae* chromosome no. 15 carrying *ARF3* and *RK11* and (B) disruption of the *RK11* gene

Open bar represents a 4.1 kb DNA fragment capable of complementing the pyridoxine auxotrophy of the mutant A22 and the arrow below it represents the direction and length of DNA sequencing. Symbols for restriction enzymes are: Bg, *Bgl*II; Bs, *Bsp*I; H, *Hind*III; N, *Nhe*I; P, *Pst*I; Sa, *Sal*I; Sc, *Sac*I; Sn, *Sna*BI; Sp, *Spe*I. The *RK11* gene was disrupted by substituting the *Spe*I–*Sna*BI fragment (588 bp) with the *URA3* marker gene (1.3 kb).

plasmid pYN1. Then the fragment was ligated into the *Xba*I–*Xma*I sites of the YCpL2 vector to give plasmid pYN4.

Construction of an *RK11* disruption fragment

For disruption of the *RK11* gene with the *URA3* gene, a fragment carrying the complete open reading frame of the *URA3* gene and its flanking regions was amplified by PCR with yeast chromosomal DNA as template and the primers 5'-ACGACCG-AGATTCCCGG-3' and 5'-CGGTAATCTCCGAACAG-3'. The PCR product of approx. 1.2 kb was cloned into pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA, U.S.A.) to give pTOPO-*URA3*. The *Bsp*EI–*Nhe*I fragment of plasmid pYN1 was inserted into the *Xma*I–*Xba*I sites of pUC18. An *Spe*I–*Sna*BI fragment (588 bp) of the resulting recombinant was substituted with a fragment of *Spe*I–*Sma*I (1260 bp), excised from pTOPO-*URA3* containing the *URA3* gene as a marker, to generate plasmid pDRKU3. A *Sac*I–*Sal*I fragment (2.6 kb) of this plasmid was used for disruption of the *RK11* gene (Figure 1B).

Diploid construction, *RK11* disruption and tetrad analysis

Pyridoxine auxotrophic mutant A22 was allowed to mate with the α mating-type strain D452-2 to give diploid Da4. The diploidal cells were then transformed with the disruption fragment prepared above to allow homologous recombination to occur on a uracil-free plate. Chromosomal DNA was isolated from the diploids obtained from the uracil-free medium and used as a template for PCR to check whether the recombination had taken place properly on the target *RK11* gene with a pair of *RK11* primers 5'-ACTACGGTTTCTGTCTTTGCC-3' (primer 1) and 5'-GATTACCCACCTGATATGCGT-3' (primer 2). Two fragments were observed for the PCR products on electrophoresis, one being identical in size with that of the wild-type gene (1.3 kb) and the other equivalent to the size (1.8 kb) of the *RK11* disrupted with *URA3* (results not shown).

The diploid cells thus prepared were checked for their pyridoxine requirements and allowed to sporulate for tetrad analysis. Thus the diploid cells were patched on to a sporulation plate and incubated for 5 days at 25 °C. For tetrad analysis, they were suspended in 50 µl of a glucuronidase (0.5 mg/ml) solution and incubated for 10 min at 37 °C. Then the tetrads were dissected and incubated for 1 or 2 days until colonies appeared. The haploid cells were examined for their uracil and pyridoxine requirements and mating type.

Identification of the site of mutation on the *RK11* gene

For isolation of the mutated *RK11* gene, genomic DNA from mutant A22 was digested with *Bgl*II and *Hind*III, and a 3.4 kb fragment was generated and isolated by agarose gel electrophoresis and cloned into the *Bam*HI and *Hind*III sites of pUC18. *E. coli* JM109 cells were transformed with the ligation mixture and the transformants were examined for the occurrence of the *RK11* gene by PCR with primers 1 and 2 to give plasmid pM34. The mutated site was identified by nucleotide sequencing for *Sna*BI–*Spe*I and *Spe*I–*Sca*I subclones prepared from pM34.

Construction of an overexpression plasmid for ribose 5-phosphate ketol-isomerase

To express yeast ribose 5-phosphate ketol-isomerase in *E. coli*, the *RK11* gene (774 bp) with and without a mutation at position 566 was amplified by PCR with the following two oligonucleotides and chromosomal DNA from wild-type yeast or mutant A22 as primers and template respectively: primer 3, 5'-ATACCATGGCTGCCGGTGTCCCAAAA-3' and primer 4, 5'-TATGGATCCTCACTTTTCGGTAACACTACC-3' (underline indicates the restriction enzyme site). After heating at 94 °C for 5 min, the following PCR cycle was repeated 25 times: 94 °C, 1 min; 55 °C, 1 min; 72 °C, 1 min and finally heated at 72 °C for 5 min. The product was cloned into pCR2.1-TOPO (Invitrogen), sequenced and then re-cloned into the *Nco*I–*Bam*HI sites of vector pET21d (Novagen, Madison, WI, U.S.A.). The resulting recombinant plasmids were termed pRK11 and pRK11m for the wild-type and the Arg¹⁸⁹ → Lys (R189K) mutant respectively.

Expression and purification of ribose 5-phosphate ketol-isomerase

E. coli BL21(DE3) (Novagen) was transformed with one of the recombinant plasmids prepared above and grown in Luria–Bertani medium in the presence of 50 µg/ml ampicillin to the late exponential phase, whereupon isopropyl β-D-thiogalactopyranoside was added to 0.4 mM. Cells from a 1 litre culture were harvested, washed with PBS, taken up in 50 ml of 120 mM KPi (pH 7.0), containing 1 mM EDTA, 1 mM dithiothreitol, 8.75 mg of PMSF and 0.25 ml of DMSO and disrupted by sonication. The homogenate was centrifuged at 17000 *g* for 20 min and the supernatant was fractionated with ammonium sulphate. The pellet obtained at 60–70 % saturation was collected, taken up in 50 mM KPi (pH 7.0) containing 0.1 mM EDTA and 0.1 mM dithiothreitol and dialysed against the same buffer. The solution was then subjected to DEAE-cellulose (2 cm × 10 cm) chromatography and the protein was eluted with a linear gradient of 0–500 mM NaCl in 50 mM KPi (pH 7.0). Ribose 5-phosphate ketol-isomerase was eluted at 200–300 mM salt. The pooled fractions (20 ml) were concentrated to 10 ml by dialysis against 15 % poly(ethylene glycol) 20000 and then subjected to gel-filtration chromatography on Superdex 200 (2.6 cm × 60 cm; Amersham

Biosciences, Piscataway, NJ, U.S.A.) to give 90 and 9 mg of the wild-type and the R189K mutant respectively. The lower yield for the mutant is due to its propensity to precipitate during purification. The N-terminal sequence of the purified protein was determined to be AAGVPKIDAL, in conformity with that deduced from the DNA sequence except for the absence of the first methionine.

Enzyme assay

The activity of ribose 5-phosphate ketol-isomerase was determined by the cysteine–carbazole method essentially as reported previously [17]. Briefly, after incubation of the reaction mixture with cysteine and carbazole in 70 % sulphuric acid for 1 h at room temperature (25 °C), absorbance was read at 560 nm. Fructose 6-phosphate was used in place of ribulose 5-phosphate for the construction of a standard curve. One unit of enzyme activity is defined as the amount of protein capable of converting 1 µmol of substrate into product in 1 min. Rates were determined from the early phase of the reaction and all the kinetic experiments were performed in triplicate. Results were subjected to non-linear regression analysis on the basis of the Michaelis–Menten formulation. Kinetic parameters K_m and k_{cat} and their standard errors were estimated from results determined over the substrate concentration ranging from 0.5 to 20 mM for the wild-type or from 0.5 to 40 mM for the mutant.

RESULTS

Yeast pyridoxine auxotrophs

Pyridoxine auxotrophs of *S. cerevisiae* were prepared by ethyl methanesulphonate mutagenesis [15]. Several strains were tested including D451-3 and D373-1, and in all cases auxotrophs showing clear pyridoxine requirements were obtained. After several rounds of screening, 23 and 19 mutants were established from D451-3 and D373-1 respectively. Mutant A22 was one of those derived from the former.

Construction of transformants

Haploid pyridoxine auxotrophs were transformed with a genomic DNA library of *S. cerevisiae* to generate transformants capable of surviving in pyridoxine-free media. Several such transformants were indeed obtained from the mutants of D451-3 and D373-1. One of them, A22t1, derived from mutant A22, harboured a plasmid (pYN1) carrying a 4.1 kb fragment of chromosome no. 15. Two known genes, *ARF3* and *RK11*, are present on it (Figure 1). The former is assigned a GTP-binding ADP-ribosylation factor and the latter ribose 5-phosphate ketol-isomerase [18,19]. To know which of them is involved in pyridoxine biosynthesis, they were subcloned separately as detailed under the Materials and methods section. The resulting recombinant plasmids carrying *RK11* and *ARF3* were termed pYN2 and pYN3 respectively. Mutant A22 was then transformed with either of these plasmids. It was found that the transformant carrying *RK11* (pYN2) was able to grow in –PN medium, whereas the one carrying *ARF3* (pYN3) was unable to grow in such a medium (Figure 2), implying that the former gene is responsible for the loss of pyridoxine auxotrophy in the transformant.

Since plasmid YEpm4 is a multiple copy vector, the possibility that *RK11* serves as a suppressor is not ruled out. To assess this possibility, similar experiments were performed with recombinant plasmid pYN4 carrying *RK11* in a single-copy vector YCpL2. As

Figure 2 Growth of D451-3 and A22 in media containing (+PN) and not containing pyridoxine (–PN)

A22, one of the pyridoxine auxotrophs derived from D451-3, harboured one of the following plasmids in +PN and –PN media: (A) D451-3 with YEpM4, (B) A22 with pYN1 (*ARF3* and *RK11*), (C) A22 with pYN2 (*RK11*), (D) A22 with YN3 (*ARF3*), (E) A22 with pYN4 (*RK11* in a single-copy vector YCpL2) and (F) A22 with YEpM4.

shown in Figure 2, the transformant was able to grow as well as the transformant with the multiple copy plasmid (pYN2) in –PN medium, confirming that *RK11* is not a suppressor but is indeed involved in pyridoxine synthesis.

RK11 disruption and tetrad analysis

To confirm that some mutation on the *RK11* gene is indeed responsible for the pyridoxine auxotrophy observed for A22, this gene was disrupted directly. In light of the fact that the *RK11*-disrupted haploid cells were inviable [19], it was disrupted in diploid cells. Thus A22 was allowed to mate with the wild-type strain D452-2 to give a diploid cell Da4, which did not show pyridoxine auxotrophy. Then the diploid cells were transformed with an *RK11*-disrupted fragment to allow homologous recombination to occur for disruption of one of the *RK11* alleles. Five of the 14 disruptants obtained, a number close to seven, were pyridoxine auxotrophic (results not shown), proving that *RK11* is the site of mutation responsible for the pyridoxine auxotrophy of A22.

After tetrad analysis on pyridoxine-requiring and -not-requiring diploids, only two haploid cells rather than four were obtained consistently from an ascus. Those from the former exhibited pyridoxine requirements, whereas those from the latter did not. Both of them exhibited uracil requirements, confirming that each of them contained the undisrupted allele of *RK11*. This phenomenon is consistent with the observation that the *RK11*-disrupted haploid cells were inviable [19], reinforcing the view that *RK11* is indeed involved in pyridoxine synthesis.

Site of mutation on *RK11*

To identify the site of mutation on the *RK11* gene, the relevant portion of the chromosomal DNA of A22 was sequenced. It was found that the guanine residue at position 566 had been converted into an adenine residue, resulting in an amino acid substitution of arginine with lysine at position 189. The arginine residue at this position seems to be conserved in ribose 5-phosphate ketol-isomerase, at least among the following ten organisms for which reliable results are available, suggesting that it plays an important role in the functioning of this protein: *Arabidopsis thaliana* (Swiss-Prot accession no. Q9ZU38), *Caenorhabditis elegans* (P41994), *Caulobacter crescentus* (Q9A5Z4), *Drosophila melanogaster* (CG30410), *E. coli* (P27252), *Lactococcus lactis* (Q9CDI7), *Mus musculus* (P47968), *Pyrococcus horikoshii* (O50083), *S. cerevisiae* (Q12189) and *Treponema pallidum* (O83625). According to the three-dimensional structure of ribose 5-phosphate ketol-isomerase from *P. horikoshii*, which has been reported recently, Arg-162, the counterpart of Arg-189 in that organism, is located in the loop from 162 to 177. This loop is thought to play a role in subunit–subunit interaction [20].

Properties of R189K mutant of ribose 5-phosphate ketol-isomerase

Both the wild-type and the R189K mutant of ribose 5-phosphate ketol-isomerase were expressed in *E. coli*, purified to homogeneity and characterized. The specific activity (k_{cat}) of wild-type enzyme was $653 \pm 7.4 \text{ s}^{-1}$ at 25°C with a K_m value of $1.78 \pm 0.08 \text{ mM}$ for ribose 5-phosphate, values nearly equivalent to those of ribose 5-phosphate ketol-isomerase from *S. cerevisiae* [21]. However, the activity of the mutant was $3.6 \pm 0.6 \text{ s}^{-1}$ or 0.6% of that of the wild-type at best, with a 13-fold increase in K_m ($23.2 \pm 7.3 \text{ mM}$). This greatly diminished activity of the R189K mutant may be explained in several ways, e.g. Arg-189 is involved directly in catalysis or alternatively it plays an important role in maintaining the proper three-dimensional structure and/or associated structure (native ribose 5-phosphate ketol-isomerase is a tetramer).

The latter possibility was assessed by gel-filtration HPLC and CD measurements. As shown in Figure 3, a single peak was observed at 17.70 min in gel-filtration HPLC for wild-type ribose 5-phosphate ketol-isomerase which probably represents the tetramer, whereas the peak was retarded to 18.83 min with the mutant. Judging from the retention time, the peak for the mutant may be assigned a dimer, demonstrating that the R189K mutant tends to dissociate under the conditions employed. A similar result was obtained in native PAGE on 12.5% gel: the mutant migrated considerably faster compared with the wild-type enzyme (results not shown).

In addition, the molar ellipticity at 208 nm of the R189K mutant was 30% lower compared with the wild-type enzyme (Figure 4), suggesting that the native three-dimensional structure of ribose 5-phosphate ketol-isomerase was partially broken by this amino acid substitution. Taken together, Arg-189 seems to participate in the maintenance of the proper three-dimensional structure of ribose 5-phosphate ketol-isomerase. Once it is replaced with lysine, very similar in many respects to arginine of all the proteinaceous amino acids, the native structure is impaired, resulting in a loss of catalytic activity.

DISCUSSION

It was demonstrated above that the gene *RK11* participates in pyridoxine synthesis in yeast. The present identification of *RK11* follows the previous identification of *pyroA* and *SOR1* (*PDX1*) as

Figure 3 Gel-filtration HPLC of purified wild-type (upper trace) and R189K mutant (lower trace) of ribose 5-phosphate ketol-isomerase on the Tosoh TSK SW3000 gel-filtration column

Figure 4 CD spectra of purified wild-type (thick trace) and R189K mutant (thin trace) of ribose 5-phosphate ketol-isomerase determined at a protein concentration of 1.5 mg/ml

participating in pyridoxine synthesis in fungi [6,7]. In addition, *PDX2* was also identified recently [8] and it was shown that its yeast homologue *SNO1* together with *SNZ1* is involved in pyridoxine synthesis by supplying ammonia for the ring nitrogen of pyridoxine [22]. The gene product of *RK11* is known as ribose 5-phosphate ketol-isomerase, which mediates interconversion of ribose 5-phosphate and ribulose 5-phosphate in the pentose phosphate pathway.

It was reported that disruption of the *RK11* gene makes the haploid yeast cells inviable [19]. This observation seems noteworthy,

as both compounds are synthesized by several separate routes. For example, ribulose 5-phosphate is synthesized from D-xylulose 5-phosphate by RPE (ribulose 5-phosphate epimerase) and from 6-phospho-D-gluconate by 6-phosphogluconate dehydrogenase. Similarly, D-ribose 5-phosphate is synthesized from glyceraldehyde 3-phosphate and sedoheptulose 7-phosphate by TKL (transketolase). Therefore the *RK11* gene and its translation product must play an essential role in the yeast cell in addition to the clear role it plays in the non-oxidative part of the pentose phosphate pathway. This seems to be the pyridoxine synthesis. In other words, ribose 5-phosphate or ribulose 5-phosphate appears to be the starting or intermediary material for pyridoxine in yeast. This is consistent with recent reports stating that a pentose or pentulose constitutes the skeleton of pyridoxine in yeast [9]. It is interesting that *RK11*, known for a long time as participating in pentose phosphate pathway and photosynthesis also, has a novel function to participate in vitamin synthesis.

The fact that mutant A22 with a mutation on the *RK11* gene exhibits pyridoxine auxotrophy suggests that neither RPE nor TKL is working sufficiently or their activity is not high enough within the yeast cells to rescue the lethal defect of the *RK11* gene, at least under the conditions employed. This notion is consistent with the observation that TKL- or RPE-disrupted haploid yeast cells are inviable in medium containing xylulose [19,23].

With these considerations, two possible candidates for the starting or intermediary material for pyridoxine in yeast may be proposed. One is ribose 5-phosphate. Genes *pyrA* and *SOR1* (*PDX1*) were identified as a suppressant to the oxidative pressure in the cells [6,24] and, under oxidative conditions, cells need to maintain a reducing environment by enhancing the pentose phosphate pathway to supply surplus NADPH [25]. Under these conditions, ribose 5-phosphate seems to be synthesized similarly to glucose 6-phosphate and ribulose 5-phosphate. Once the *RK11* gene is disrupted, ribulose 5-phosphate is never converted into ribose 5-phosphate, resulting in a shortage of ribose 5-phosphate and a halt to pyridoxine synthesis. However, the synthesis of tryptophan, purine and pyrimidine would have been affected as well by the disruption of the *RK11* gene, as ribose 5-phosphate is their key starting material. This phenotype was not observed for mutant A22, ruling out the possibility that ribose 5-phosphate is the precursor of pyridoxine.

An alternative possibility is ribulose 5-phosphate as the starting or intermediate material. Where the intracellular NADPH level is high, the oxidative decarboxylation of gluconate 6-phosphate is repressed and the non-redox part of the pentose phosphate pathway is believed to proceed backwards [26]. If the *RK11* gene is disrupted under such circumstances, ribulose 5-phosphate will not be supplied from ribose 5-phosphate and pyridoxine synthesis will be stopped, but the synthesis of tryptophan and nucleic bases will not be affected. The phenotype of mutant A22 is consistent with this notion. It is noted that the structure of ribulose 5-phosphate conforms neatly to the 2'-4' portion of 2'-hydroxypyridoxine and, on the basis of this assumption, a putative pathway for pyridoxine synthesis is depicted in Scheme 1.

It should be noted that total disruption of the *RK11* gene renders the haploid yeast cells inviable [19], whereas the single amino acid substitution of Arg-189 with lysine makes them pyridoxine auxotrophic, suggesting that not only the function of the enzyme but also the ribose 5-phosphate ketol-isomerase protein itself is essential for the pyridoxine synthesis and eventually the sustenance of yeast cells. This may happen presumably because ribose 5-phosphate ketol-isomerase interacts with other proteins in the cell and even an inactive protein may be useful as long as it retains protein-binding capability. In fact, it was shown recently through two-hybrid analysis that ribose 5-phosphate

Scheme 1 Putative synthetic pathway to pyridoxine in yeast

The synthesis starts with ribulose 5-phosphate, which eventually constitutes the 2'–4' portion of the pyridoxine skeleton. Ammonia released from glutamine residue by the complex of Sno1p and Snz1p is incorporated into the ring, probably after condensation with an unknown component marked by '?'. This process is supposed to be mediated by Snz1p. Circled P, phosphoryl group.

ketol-isomerase interacts with an unknown protein encoded by YLL012W [27]. It is probable that the latter protein is also involved at some stage of pyridoxine synthesis. It should also be noted that in the Calvin cycle of photosynthesis, ribose 5-phosphate ketol-isomerase associates with other proteins to form a CO₂-fixing multi-enzyme complex in chloroplasts of plants [28].

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