YLR209c Encodes *Saccharomyces cerevisiae* Purine Nucleoside Phosphorylase

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The yeast *YLR209c* (*PNP1*) gene encodes a protein highly similar to purine nucleoside phosphorylases. This protein specifically metabolized inosine and guanosine. Disruption of *PNP1* led to inosine and guanosine excretion in the medium, thus showing that *PNP1* plays an important role in the metabolism of these purine nucleosides in vivo.

Purine salvage is a complex pathway allowing interconversion of bases, nucleosides, and nucleotides. In yeast, major attention has been paid to the conversion of bases into nucleotides by phosphoribosyltransferases (PRTs): adenine-PRT, hypoxanthine-guanine-PRT, and xanthine-PRT activities have been reported (19, 20), and the cognate genes have been identified (1, 5, 6). Yeast purine nucleoside metabolism has received far less attention, and only very recently was the first yeast gene encoding a purine nucleoside metabolizing enzyme identified (11). This gene, named *ADO1*, encodes adenosine kinase allowing synthesis of AMP from adenosine. Although several other enzymatic activities involved in yeast purine nucleoside metabolism have been described in the past, the corresponding genes have not yet been identified. Enzymatic activities responsible for the synthesis of inosine either from adenosine by adenosine deaminase (14) or from IMP by an IMP-specific 5′ nucleotidase have been reported (8). Also, two distinct enzymatic activities (purine nucleoside hydrolase and purine nucleoside phosphorylase [PNP]) responsible for the degradation of inosine into hypoxanthine have been reported (7). The latter two enzymes catalyze the conversion of nucleosides to bases, although through distinct enzymatic mechanisms: (i) for nucleoside hydrolase, nucleoside + $H_2O \rightarrow$ base + ribose and (ii) for nucleoside phosphorylase, nucleoside + $P_i \rightarrow$ base + ribose-1P.

As a further step toward understanding yeast purine nucleoside metabolism, we searched for open reading frames (ORFs) in the complete yeast genome sequence that would encode candidate PNP. We found an uncharacterized ORF (*YLR209c*) that encodes a putative polypeptide highly similar to human and bovine PNP (Fig. 1). This enzyme has been thoroughly studied, and the three-dimensional structures of the trimeric human and bovine PNPs have been solved (3, 10). Important residues for substrate binding and catalysis have been identified (4, 12), all of which (except Val263) are conserved in the yeast enzyme (shown by asterisks in Fig. 1).

To gain insight into the precise function of the yeast ORF *YLR209c*, the protein encoded by this ORF was tagged with 10 histidine residues at its N terminus and expressed in *Escherichia coli*. The PNP expression plasmid was constructed as follows. The *YLR209c* ORF was amplified by PCR from S288c genomic DNA with the following synthetic oligonucleotides: 359 (5′-CGATGCTCGAGATGAGTGATATCTTGAACGT-3′) and 360 (5′-GGACCCGGGTTATAATTCCCCCATTAC GG-3′). The amplification product was then digested with *Xho*I and *Sma*I and inserted in the pJC20-HisN vector (15) digested with *Xho*I and *Sma*I. The BL21(DE3) *E. coli* strain carrying the resulting plasmid was induced at 25°C for 6.5 h in the presence of 0.1 mM IPTG (isopropyl-β-D- thiogalactopyranoside). The His-tagged PNP was then purified by following the native protein purification protocol from the QIAexpressionist kit (Qiagen), with the following two modifications: phosphate buffers were replaced by Tris-HCl, and elution was done using 300 mM imidazole. The purified protein was then assayed for its ability to metabolize the various purine and pyrimidine nucleosides. In this assay, the reaction was performed at 30°C and stopped by freezing the samples after 30 min. The disappearance of the substrates (present at a concentration of 20 μM) and the appearance of the products were monitored by high-pressure liquid chromatography (HPLC) as described previously (11).

Ylr209cp was unable to metabolize the purine nucleosides adenosine and xanthosine or the pyrimidine nucleosides uridine and cytidine (Fig. 2, middle and right panels), while inosine and guanosine were substrates for Ylr209p (Fig. 2, left panel). Therefore, the *YLR209c* ORF clearly encodes a PNP and was renamed *PNP1*. The yeast PNP is thus closely related to the human enzyme both structurally (50% sequence identity) and functionally, since both enzymes only accept inosine and guanosine as substrates. In contrast, yeast PNP appears more distant to the *E. coli* enzyme (<10% identity), which is hexameric (13) and also accepts adenosine as a substrate (9).

The PNP activity was then measured using a coupled reaction with xanthine oxidase. The principle of the assay is that hypoxanthine produced from degradation of inosine by PNP is followed by its two-step transformation into uric acid by commercial (Roche 110442) xanthine oxidase (0.5 U/reaction). Ap-

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FIG. 1. Sequence comparison of PNPs from various species. The amino acid sequence deduced from the nucleotide sequence of the *YLR209c* ORF (*S. cerevisiae*) was compared with the PNP sequences from *Homo sapiens* (18) and *Bos taurus* (2). Identities and similarities are highlighted in black and gray boxes, respectively. Dashes indicate the gaps created for alignment. Asterisks indicate residues important for substrate binding and catalysis.

pearance of uric acid in the reaction is then monitored by its specific absorbance at 293 nm ($\varepsilon = 12$ mM^{-1} cm^{-1}). The reaction was done at 25°C in potassium phosphate buffer (pH 7.5; 100mM) with 33 ng of purified PNP enzyme in a final volume of 0.8 ml. Using this assay, the K_m for inosine was found to be 166 ± 8 μM (Fig. 3A). Addition of adenosine (up to 800 μM), xanthosine (up to 600 μM), uridine (up to 800 μM), and cytidine (up to 400 μM) did not result in any detectable inhibition of Pnp1p activity measured using inosine as the substrate at a final concentration of 175 μM. In contrast, guanosine had a very strong competitive inhibitory effect, and its K_i was estimated at 8.4 ± 2.7 μM (Fig. 3B). This value also

refers to the K_m for guanosine since this substance is a strict competitive inhibitor of inosine (Fig. 3B). Guanosine can therefore be bound more efficiently than inosine by yeast PNP, a situation similar to that reported for human PNP for which the K_ms for guanosine and inosine are 12 and 45 μM, respectively (17). Finally, the V_{max} and k_{cat} of PNP, with inosine as substrate, were 2.6 μM min^{-1} and 38 s^{-1}, respectively. This latter parameter of the yeast enzyme is therefore similar to the one reported for human PNP and inosine: 57 s^{-1} for k_{cat} (17).

A *pnp1* null mutant (purchased from Euroscarf) did not show any obvious growth defect. We reasoned that if PNP activity is important for purine metabolism, the *pnp1* mutant
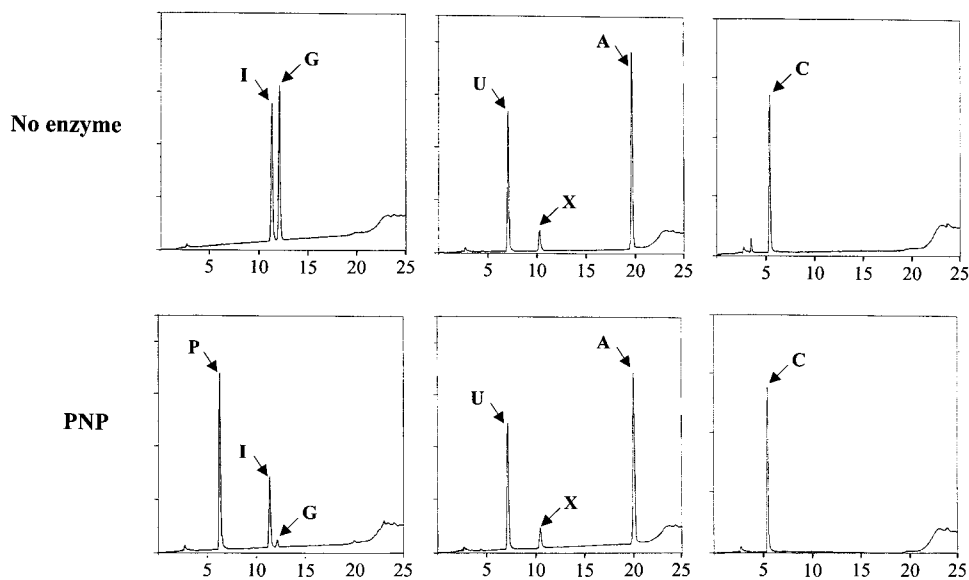

FIG. 2. Substrate specificity of the yeast PNP. The yeast PNP purified from *E. coli* was assayed for catalysis of various purine and pyrimidine nucleosides (inosine + guanosine, left panels; uridine + xanthosine + adenosine, middle panels; cytidine, right panels). Abbreviations: A, adenosine; C, cytidine; G, guanosine; I, inosine; P, hypoxanthine + guanine; U, uridine. The nucleosides treated with the enzyme (bottom panels) or untreated (top panels) were separated by HPLC.
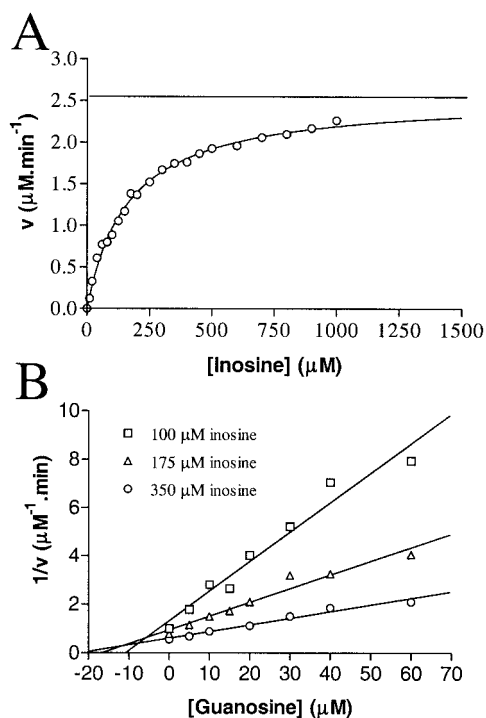
FIG. 3. Kinetic parameters of the yeast purine nucleoside phosphorylase. (A) Determination of the K_m for inosine using a xanthine oxidase-coupled assay. V_{max} (horizontal line) and K_m were calculated by nonlinear regression analysis of the saturation curve. (B) The K_i for guanosine was determined by linear regression of the data presented in the Dixon plot.
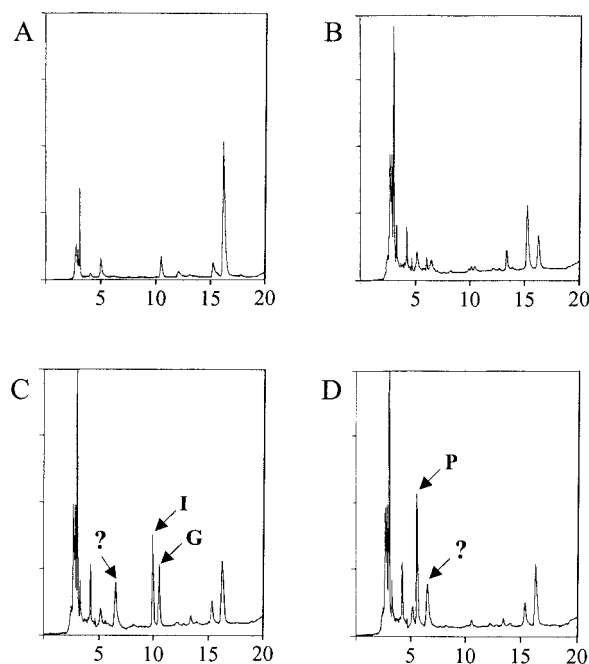

FIG. 4. Excretion of inosine and guanosine by the *pnp1* mutant. Purine derivatives in the growth medium were separated by HPLC. Compound separation was monitored by following absorbance at 260 nm. HPLC profiles are presented as a function of retention time on the column. (A) SC medium. (B) SC medium after growth of the wild-type strain (BY4741). (C) SC medium after growth of the *pnp1* mutant strain (Y04158). (D) Same medium as in panel C but treated with commercial PNP. Arrows indicate the peaks specifically found in the *pnp1* growth medium. Abbreviations: G, guanosine; I, inosine; P, hypoxanthine + guanine. Unidentified peaks are indicated by a question mark.

should accumulate the natural substrates of the enzyme and that some of them could be excreted in the growth medium. Wild-type (BY4741: *MATa* ura3Δ0 leu2Δ0 met15Δ0 his3Δ1) and *pnp1* (Y04158:*MATa* ura3Δ0 leu2Δ0 met15Δ0 his3Δ1 ylr209c::KanMX4) strains were grown in adenine- and uracil-free SC medium (16) to mid-exponential phase. Cells were then harvested, and the medium was filtered through a 0.2-μm (pore-size) filter. Separation of compounds in the growth medium was done by chromatography as described previously (11). The inosine and guanosine peaks in the *pnp1* strain growth medium were identified by their respective retention times and confirmed by treating the growth medium with commercial *E. coli* PNP as recommended by the supplier (Sigma). Analysis of the medium after growth of the wild-type and mutant *pnp1* strains did indeed reveal that the mutant strain excretes both inosine and guanosine in the medium (Fig. 4). This result strongly supports the idea that inosine and guanosine are the natural substrates of yeast PNP in vivo.

Inosine and guanosine excretion by the *pnp1* mutant also suggests that the alternative purine nucleoside hydrolase activity described by Heppel and Hilmoe (7) cannot compensate for the nucleoside accumulation due to the lack of PNP. Our attempt to identify a candidate ORF for such a purine nucleoside hydrolase activity was unsuccessful. The best candidate for inosine hydrolase was ORF *YDR400w*, which was found to encode a cytidine-uridine hydrolase but could not metabolize any of the purine nucleosides (K.L. and B.D.-F., unpublished results). The identification of *PNP1* further improves our un-

derstanding of purine metabolism in yeast, although several genes encoding purine nucleoside-metabolizing enzymes remain to be identified.

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