Purification, Molecular Cloning, and Catalytic Activity of Schizosaccharomyces pombe Pyridoxal Reductase

A POSSIBLE ADDITIONAL FAMILY IN THE ALDO-KETO REDUCTASE SUPERFAMILY*

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Pyridoxal reductase (PL reductase), which catalyzes reduction of PL by NADPH to form pyridoxine and NADP⁺, was purified from Schizosaccharomyces pombe. The purified enzyme was very unstable but was stabilized by low concentrations of various detergents such as Tween 40. The enzyme was a monomeric protein with the native molecular weight of 41,000 ± 1,600. The enzyme showed a single absorption peak at 280 nm (ε280 = 10.0). PL and 2-nitrobenzaldehyde were excellent substrates, and no measurable activity was observed with short chain aliphatic aldehydes; substrate specificity of PL reductase was obviously different from those of yeast aldo-keto reductases (AKRs) so far purified. The peptide sequences of PL reductase were identical with those in a hypothetical 333-amino acid protein from S. pombe (the DDBJ/EMBL/GenBank™ accession number D89205). The gene corresponding to this protein was expressed in Escherichia coli, and the purified protein was found to have PL reductase activity. The recombinant PL reductase showed the same properties as those of native PL reductase. PL reductase showed only low sequence identities with members of AKR superfamily established to date; it shows the highest identity (18.5%) with human Shaker-related voltage-gated K⁺ channel β2 subunit. The elements of secondary structure of PL reductase, however, distributed similarly to those demonstrated in the three-dimensional structure of human aldose reductase except that loop A region is lost, and loop B region is extended. Amino acid residues involved in substrate binding or catalysis are also conserved. Conservation of these features, together with the major modifications, establish PL reductase as the first member of a new AKR family, AKR8.

Pyridoxal reductase (PL¹ reductase) (formerly designated as PN dehydrogenase) catalyzes reduction of PL with NADPH and oxidation of PN with NADP⁺ as the reverse reaction. The enzyme was for the first time found in a budding yeast, Saccharomyces cerevisiae, i.e. bakers’ (¹) and brewers’ (²) yeasts. Girard and Snell (³) have purified it to homogeneity from brewers’ yeast and showed that the enzyme is a monomeric protein with the molecular weight of about 33,000. They designated the enzyme as PL reductase because of the equilibrium of the enzyme reaction lying so far to formation of PN, and the substrate specificity, molecular weight, and the monomeric structure of the enzyme. The enzyme resembled chlordecone reductase (⁴) in optimal pH, molecular weight, specific requirement of for NADPH, and behavior toward sulfhydryl reagents, barbital, and common ketone substrates. The chlordecone reductase from human liver belongs to family 1 of the AKR superfamily.² The AKRs form an expanding oxidoreductase superfamily classified into seven families containing a variety of monomeric oxidoreductases such as aldehyde and aldose reductases, hydroxysteroid dehydrogenases, chalcone reductases, Shaker-related voltage-gated K⁺ channel β2 subunits, and aflatoxin B1-aldehyde reductases (⁵). Because the nomenclature system is based on identities in amino acid sequence (⁵), elucidation of primary structure of PL reductase is required to classify the enzyme in the family.

We recently found that a fission yeast, Schizosaccharomyces pombe, also contained PL reductase activity, suggesting that the enzyme also plays an important role in other eukaryotes. Here we show the purification and properties of PL reductase from S. pombe. Primary structure of the enzyme was determined based on cloning and expression of its gene in Escherichia coli. The results showed that the enzyme is the founding member of the 8th AKR family.

EXPERIMENTAL PROCEDURES

Materials

NAD⁺, NADP⁺, NADH, NADPH, and a Cosmosil C₁₈ column were purchased from NaCaLBiTes, Kyoto, Japan. PL, PN, and PLP were from Wako Chemicals, Osaka, Japan. Matrix Orange A was purchased from Grace Japan, Amicon, Tokyo, Japan. Butyl-Toyopearl and DEAE-Toyopearl were from Tosoh Corp., Tokyo, Japan. An Ultra-Free concentrator was from Milipore, Tokyo, Japan. A [lys] endopeptidase was from Roche Molecular Biochemicals, Mannheim, Germany. Primer DNAs for PCR were from Kurabo, Osaka, Japan. Restriction enzymes, E. coli JM109 competent cells, a Takara LA-PCR buffer II (Mg²⁺ plus), a Takara LA Taq polymerase, and a SUPREC-01 tube were from Takara Shuzo, Kyoto, Japan. A Ligation High was from Toyobo, Osaka, Japan. A plasmid pTrc99A was from Amersham Pharmacia Biotech, Uppsala, Sweden. A plasmid SY1115 carrying the PL reductase gene of S. pombe

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The nucleotide sequence reported in this paper has been submitted to the DDBJ/GenBank™/EBI Data Bank with the accession number D89205.

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‡ The abbreviations used are: PL, pyridoxal; AKR, aldo-keto reductase; PN, pyridoxine; PLP, pyridoxal 5-phosphate; MOPS, 3-(N-morpholin­o)propanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; 2-CBA, 2-carboxylbenzaldehyde; PCR, polymerase chain reaction; kbp, kilobase pair; bp, base pair.

In order to determine the primary structure of the enzyme, the nucleotide sequence was determined based on cloning and expression of its gene in E. coli JM109 competent cells. A Takara LA-PCR buffer II (Mg²⁺ plus), a Takara LA Taq polymerase, and a SUPREC-01 tube were from Takara Shuzo, Kyoto, Japan. A Ligation High was from Toyobo, Osaka, Japan. A plasmid pTrc99A was from Amersham Pharmacia Biotech, Uppsala, Sweden. A plasmid SY1115 carrying the PL reductase gene of S. pombe

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23185
TABLE I

Purification of PL reductase from S. pombe

| Steps                | Total protein (mg) | Total activity (units) | Specific activity (units/g) | Yield (%) |
|----------------------|--------------------|------------------------|-----------------------------|-----------|
| Crude extract        | 112.38             | 28.74                  | 0.26                        | 100       |
| Butyl-Toyopearl      | 4.48               | 16.87                  | 3.76                        | 58.7      |
| Orange A             | 0.42               | 5.43                   | 12.93                       | 18.9      |
| DEAE-Toyopearl       | 0.07               | 1.91                   | 27.2                        | 6.6       |

was a kind gift from Dr. Yoshioka of the ex-Okyama project, Japan. All other chemicals were of analytical grade.

Microorganism and the Culture Conditions

*S. pombe* IFO 0346 cells were grown at 30 °C for 72 h with shaking in a synthetic medium containing 1% n-glucose, 100 mM PN, and yeast nitrogen base, containing neither amino acids nor thiamine. The cells were harvested by centrifugation at 10,000 x g for 10 min, washed twice with 0.9% NaCl, and then stored at -20 °C.

Enzyme Assays

PL reductase was assayed by measuring the initial decrease in A_{340} of NADPH at 37 °C in 1.0 ml of the reaction mixture. Each reaction mixture contained 0.2 mM PL, 0.2 mM NADPH, 0.1 mM MOPS/KOH buffer (pH 7.5), and the enzyme. The reaction was started by addition of PL. One unit of activity was defined as the amount of enzyme required to reduce 1 pmol of PL per min.

Enzyme Purification

All the steps were performed at 4–10 °C.

Step 1—Frozen cells (195 g, wet weight) were thawed and suspended in 390 ml of Buffer C, 100 mM sodium citrate (pH 6.5) containing stabilizing reagents (1 mM PMSF, 0.01% 2-mercaptoethanol, 1 mM EDTA, and 0.1 mM PL). The suspension (100 ml, each) was vigorously vortexed for 1 min and centrifuged at 10,000 x g for 10 min to separate the supernatant solution. The precipitated cells were resuspended in 390 ml of Buffer C, vortexed, and centrifuged. The supernatant solution was applied to butyl-Toyopearl column (1.8 x 40 cm). The enzyme was eluted with Buffer M containing 1 mM NADPH. The 1.2-kbp fragment carrying the PL reductase gene (pTR + ) was cut out from a plasmid SY1115 with both EcoRI and KpnI, collected using a SUPREC-01 tube, and then ligated into the EcoRI-KpnI site of pTrc99A. The recombinant plasmid was designated as pPLR1. The PL reductase-coding sequence in pPLR1 was amplified by PCR using the two oligonucleotides as the primers: primer NF (sense) and 5'-GCGAAGCTTGGTACCAGGAGAAGATGCTATGTTAGGAGATTTAA-3' (primer NF, sense) and 5'-GCGG-GATCCTTAACCCGGAAGATGTCGCCCAAGGCTGTTCACTTCAATTTG (primer CR, antisense) to introduce BamHI (underlined in primer NF), ribosome-binding (boldface types in primer NF), and HindIII sites (underlined in primer CR). The reaction mixture (50 µl) of PCR consisted of Takara LA-PCR buffer II (Mg²⁺ plus), 20 nmol of each dNTP, 2.5 units of Takara LA Taq polymerase, 0.5 mg of a plasmid pPLR1 (as a template), and 100 pmol of each primer. The mixture was heated at 98 °C for 20 s and then incubated at 68 °C for 20 min. The programmed temperature shift was repeated 16 times, and then, without delay, “autosegment extension” program was done as follows: heating at 98 °C for 20 s and incubation at 68 °C for 20 min + t s, where t denotes the segment extension time that increases by 15 s each cycle; the temperature shift was repeated 14 times. Finally, the mixture was held at 72 °C for 10 min. The amplified DNA fragments (about 1.0 kb) were digested by BamHI and HindIII and then ligated into the BamHI-HindIII site of pTrc99A. The constructed plasmid was designated as pPLR2. A plasmid pPLR3 carrying a modified pTR + was disrupted by substitutions of certain nucleotides without changing the amino acid sequence of PL reductase, was prepared as follows. The 669-bp fragment of *S. pombe* Pyridoxal Reductase gene was amplified by PCR using the two oligonucleotides as the primers: primer NF (sense) and 5'-GCGG-GATCCTTAACCCGGAAGATGTCGCCCAAGGCTGTTCACTTCAATTTG (primer CR, antisense) to introduce BamHI and HindIII sites (underlined in primer MR). PCR was performed

FIG. 1. SDS-PAGE of crude and purified preparations of PL reductase. Lane A, crude extract (19.3 μg of protein); lane B, a purified fraction (13.8 μg) from butyl-Toyopearl column; lane C, the fraction (2.5 μg) from Orange A column; and lane D, the fraction (2.1 μg) from DEAE-Toyopearl column. Molecular weight markers (0.5 μg, each) were rabbit muscle phosphorylase (M₉ = 97,400), bovine serum albumin (66,200), hen egg white ovalbumin (45,000), bovine carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and hen egg white lysozyme (14,400).
**TABLE II**

Substrate specificity of PL reductase from *S. pombe*

| Substrate                | $K_m$  | $k_{cat}$ | $k_{cat}/K_m$ |
|--------------------------|--------|-----------|---------------|
| Pyridoxal                | 0.909  | 56.1      | 61.7          |
| Pyridoxal 5'-phosphate   | NMA    |           |               |
| 2-Nitrobenzaldehyde      | 0.096  | 33.6      | 350           |
| 3-Nitrobenzaldehyde      | NMA    |           |               |
| 4-Nitrobenzaldehyde      | 0.012  | 0.196     | 16.3          |
| 5-Nitrosalicylaldehyde   | NMA    |           |               |
| 2-Carboxybenzaldehyde    | NMA    |           |               |
| Benzaldehyde             | NMA    |           |               |
| 2-Phthalaldehyde         | 0.118  | 1.28      | 10.8          |
| 3-Phthalaldehyde         | NMA    |           |               |
| 4-Phthalaldehyde         | NMA    |           |               |
| Pyridine-2-aldehyde      | 1.31   | 15.1      | 11.5          |
| Pyridine-3-aldehyde      | 0.349  | 0.203     | 0.581         |
| Pyridine-4-aldehyde      | 1.13   | 1.78      | 1.57          |
| Succinic semialdehyde    | NMA    |           |               |
| 9,10-Phenanthrenequinone | NMA    |           |               |
| Isatin                   | NMA    |           |               |
| Isovaleraldehyde         | NMA    |           |               |
| Acetaldehyde             | NMA    |           |               |
| Formaldehyde             | NMA    |           |               |

* NMA, no measurable activity.

as described above. The 333-bp fragment of *plr* was encoding the carboxy-terminal 110 amino acids of PL reductase and containing the stop codon, was amplified by PCR using two primer oligonucleotides: 5'-G-GCGCTRTTCCCCGTTTCCGGAAATTTTGGCAGATTTAGCCTGTATTT-3' (primer MF, sense), in which substituted nucleotides are shown as boldface and EcoRI site to be introduced is underlined, and the primer CR. PCR was performed under the same conditions. Both the amplified DNA fragments were digested by EcoRI and then ligated. The ligated fragment (about 1.0 kbp) was electrophoresed on an agarose gel and then ligated. The ligated DNA fragment was inserted into the BamHI-HindIII site of pTrc99A to obtain pPLR3. The nucleotide sequences of these genes were verified using an Applied Biosystems 373A DNA sequencer with a PRISM kit (Perkin-Elmer).

The plasmids pPLR1, pPLR2, or pPLR3 were introduced into Takara *E. coli* JM109-competent cells. The clone cells were used for analysis of the enzyme production in *E. coli*.

**Purification of Recombinant PL Reductase**

Cells of *E. coli* JM109 clone harboring pPLR3 were grown in 5 liters of LB medium (7) containing ampicillin (50 μg/ml) and isopropyl-β-D-thiogalactopyranoside (1 mM) at 37 °C for 16 h. The wet weight (5 g) was harvested and disrupted by sonication to prepare crude extract. PL reductase (5.3 mg of total protein; 110.6 units of total activity) was purified from the crude extracts (2,100 mg of total protein; 18.9 g) were harvested and disrupted by sonication to prepare crude extract. The enzyme was purified to homogeneity by three steps of column chromatography (Table I). Almost all of the enzyme activity was extracted from the frozen yeast cells, suggesting different cellular localization of the two enzymes. PL reductase was stable in a buffer containing 1 mM MOPS/KOH (pH 7.5) containing 1.0 mM EDTA, 0.1 mM PMSF, 0.01% 2-mercaptoethanol, and 0.005% Tween 40; $K_m$ was 10.0. PL reductase showed a single absorption maximum at 280 nm when it was excited at 280 nm; the fluorescence intensity was decreased in the presence of NADPH. One Trp residue in

**Homology Search of PL Reductase with Other Proteins**

DNA and protein database in the DDBJ/EMBL/GenBank™ were searched for proteins homologous with PL reductase by use of the BLAST algorithm (8).

**RESULTS**

**Purification and Some Properties of PL Reductase—**The enzyme was purified to homogeneity by three steps of column chromatography (Table I). Almost all of the enzyme activity was eluted from the frozen yeast cells, but not from intact yeast cells, by the vigorous mixing. On the contrary, lactate dehydrogenase (a cytosolic enzyme) activity was not extracted from the frozen yeast cells, suggesting different cellular localization of the two enzymes. PL reductase was stable in a buffer without a detergent before the butyl-Toyopearl column chromatography. However, after the step, the enzyme became quite unstable; 95.2% of original activity was lost after 24 h when stored at 4 °C. Very low concentrations of several detergents stabilized the enzyme; 0.005% of Tween 40 was more effective at this concentration than Triton X-100, Tween 20, or Tween 80.

The purified enzyme showed a single protein band with a molecular weight of 37,600 ± 370 (average and S.D. of four experiments) on an SDS-PAGE gel (Fig. 1). The molecular weight of the native enzyme was 41,000 ± 1,600 (average and S.D. of three experiments) by gel filtration. The enzyme is thus a monomeric protein.

The optimum pH for PN formation was 6.5–7.5; PL reductase was not active at pH 5.0, but 17% of the maximal activity was displayed at pH 8.5. The optimum pH for PL formation was 7.5–8.5; the enzyme was not active at pH 6.5, but 18% of the maximal activity was displayed at pH 10.0. NADPH ($K_m$ = 16 ± 0.8 mM at pH 7.5) and NADP$^+$ were required for PN and PL formations, respectively; NADH and NAD$^+$ were inactive as coenzymes.

Spectroscopic Properties—Between 250 and 700 nm, PL reductase showed a single absorption maximum at 280 nm in 10 mM MOPS/KOH (pH 7.5) containing 1.0 mM EDTA, 0.1 mM PMSF, 0.01% 2-mercaptoethanol, and 0.005% Tween 40; $E_{1%}$ was 10.0. PL reductase showed an emission maximum at 330 nm when it was excited at 280 nm; the fluorescence intensity was decreased in the presence of NADPH. One Trp residue in
the enzyme may be involved in the interaction of the enzyme and NADPH.

**Substrate Specificity**—Steady-state kinetic parameters for PL reductase are given in Table II. All substrates showed Michaelis-Menten kinetics in the concentration range studied. PL and 2-nitrobenzaldehyde were excellent substrates. Although PL was a substrate with the highest \( k_{\text{cat}} \), \( K_m \) was 9.4-fold higher than that of 2-nitrobenzaldehyde; the hemeicetal form of PL, which is predominant at around pH 7.0, may not be used as a substrate. Thus, 2-methyl and 3-hydroxy groups and ring nitrogen in PL are not essential for high substrate activity. In contrast, the 5-hydroxymethyl group in PL and the nitro group (but not the carboxyl group) adjacent to the formyl group in 2-benzaldehyde were essential: pyridine-4-aldehyde was a poor substrate, and benzaldehyde or 2-carboxybenzaldehyde was not substrate. 2-Phthalaldehyde was a moderate substrate and gave Michaelis-Menten kinetics when initial reaction rates were measured. However, PL reductase was inactivated during reduction of 2-phthalaldehyde by an unknown reason. No measurable activity was observed with short chain aliphatic aldehydes.

**Amino Acid Sequences of Peptides of PL Reductase and Search for the Enzyme Gene**—The amino-terminal sequence could not be determined by Edman degradation probably because an amino-terminal amino acid residue is modified. Then, we determined the amino acid sequences of lysylendopeptidase and cydnogen bromide peptides of the enzyme (shown by underlines in Fig. 2). Homology search was performed with these sequences. Sequences of a hypothetical protein of *S. pombe* composed of 333 amino acids (the DDBJ/EMBL/GenBank™ accession number D89205) agreed well with that of the enzyme in the crude extract of *E. coli* JM109/pPLR3. The derived clone harboring pPLR3 (see “Experimental Procedures”), the derived clone harboring pPLR3 (*E. coli* JM109/pPLR3) produced high levels of PL reductase; an average specific activity was 0.78 unit/mg. Thus, the palindrome structure in plr*"* appears responsible for the low expression in *E. coli*.

**Properties of Recombinant PL Reductase**—PL reductase from *E. coli* JM109/pPLR3 showed the same enzymatic properties, such as the molecular weight, specific activity, and amino-terminal 12 amino acids sequence, as the enzyme from *S. pombe*.

**Sequence Comparisons**—The amino acid sequence of PL reductase was compared with other protein sequences. Unexpectedly, no proteins belonging to the AKR superfamily showed higher than 20% homology. However, 12 proteins, whose functions are unknown, were found to have higher identity than 20% (Table III). The F8A5.2 protein of *Arabidopsis thaliana* showed the highest identity; this protein is a auxin-induced protein. YAK protein of *S. pombe* was similar to PL reductase, suggesting it is an isoenzyme of PL reductase. IolS protein of *Bacillus subtilis* is encoded by a gene which is a member of operon of myo-inositol degradation in the bacterium (9), but its function is unknown.

**Secondary Structure Comparisons**—Although the primary structure of PL reductase showed low identity with the AKRs so far reported, a secondary structure predicted with a Genetyx software (Software Development, Tokyo, Japan) was similar to that of human aldose reductase (10, 11), a typical member of AKRs. Fig. 4 shows the alignment of PL reductase, human *Shaker*-related voltage-gated K*"* channel 

\[ \text{specific activity (U/mg)} \]

![Graph](image)

**Fig. 3. Recombinant plasmids and expression of PL reductase in *E. coli*. A, schematic structures of pPLR1, pPLR2, and pPLR3, prepared as described under “Experimental Procedures.” Pvec, *trc* promoter; SD, Shine-Dargarno sequence. B, expression of PL reductase activity. 10 colonies of *E. coli* JM109/pTrc99A, JM109/pPLR1, JM109/pPLR2, and JM109/pPLR3 clones were isolated. They were individually grown in 100 ml of LB medium (7) containing 50 µg/ml ampicillin and 1 mlt isopropyl-β-1-thiogalactopyranoside. PL reductase activity in the crude extracts of the cells (0.2 g, wet weight) was measured. An average and standard deviation of the specific activity is shown.
DISCUSSION

The complete amino acid sequences of three kinds of yeast AKRs have been reported so far: xylose reductases (aldose reductases) from xylose-assimilating yeasts (15, 16), aldehyde reductase from *Sporobolomyces salmonicolar* (17), and an enzyme from *S. cerevisiae* which shows high reactivity toward α- and β-keto esters (18, 19). An amino-terminal sequence of AKR from *S. cerevisiae* has been reported (20). PL reductase showed less than 10% identity with these yeast AKRs and differs substantially from them in substrate specificity. For example, PL reductase did not reduce aldoses such as xylose, and in contrast to AKRs from *S. salmonicolar* and *S. cerevisiae*, which show high activity toward 4-nitrobenzaldehyde (17, 19, 20), PL reductase showed the highest reactivity toward 2-nitrobenzaldehyde. Thus, the previously studied yeast AKRs may show little if any activity toward PL although their activity toward PL has not been reported. It will be interesting to determine whether PL reductase distributes among yeasts as aldose reductase.

The predicted secondary structure of PL reductase suggested that the enzyme adopts a fold similar to that of human aldose reductase, whose structure is known (10, 11). The alignment showed that important catalytic, NADPH-binding residues were aligned with putative equivalent residues in AKRs. Asp-

| Source     | Abbreviation | Accession | Length | Identity | Similarity |
|------------|--------------|-----------|--------|----------|------------|
| A. thaliana| F8A520       | AC002292* | 340    | 37       | 55         |
| Helianthus annuus | HaAc1 | AF03031* | 338    | 34       | 55         |
| S. pombe   | YAK°         | Q09923°   | 340    | 34       | 52         |
| *S. cerevisiae* | Ypr127wp | U40825°   | 345    | 34       | 53         |
| Nicotiana tabacum | NTAUCX115 | X56267° | 307    | 33       | 53         |
| Zea mays   | IN2–2       | F49294°   | 306    | 36       | 54         |
| *Synechocystis* sp. | SYCSSLIIH | D64006° | 314    | 31       | 50         |
| B. subtilis| GSP69       | P80674°   | 331    | 27       | 49         |
| B. subtilis| YccK        | AB000617* | 310    | 26       | 43         |
| E. coli    | Io1S        | D98820°   | 326    | 26       | 49         |
| E. coli    | e346        | AE000382° | 346    | 24       | 47         |
| B. subtilis| Io1S        | D96846°   | 310    | 24       | 43         |

a DDBJ/EMBL/GenBank™. b Swiss-Prot.

**FIG. 4.** Predicted secondary structural elements of PL reductase and some other AKRs. Amino acid residues comprising α-helix and β-sheet in PL reductase are shown by a solid line and α and β, respectively. The sequence was aligned with that of human aldose reductase (hADR), whose secondary structural elements had been determined by x-ray crystallography; its α-helix and β-sheet structures are shown as H1–H10 and S1–S10, respectively. The amino acid sequences of human *Shaker*-related voltage-gated K⁺ channel β₂-subunit (hKCH) and human 2-CBA reductase (hCBR) were also aligned; their secondary elements are not shown. Gaps were introduced to maximize identity using the method of Dayhoff et al. (24). An amino acid residue common in 3 or 4 sequences is shown by boldface type. The numbering of the amino acid residues of hADR (shown below sequence) excludes the amino-terminal methionine, whereas the methionine was numbered as the first residue in that of PL reductase, hKCH, and hCBR.

**TABLE III**

Hypothetical proteins similar to PL reductase from *S. pombe*

In the column headings, Accession indicates the accession numbers. Length is the number of amino acid residues of the hypothetical protein. The corresponding sequence data are indicated in footnotes a and b. Scores of Identity and Similarity of the hypothetical protein with PL reductase were obtained by the BLAST search in the DDBJ Homology Search System.

| Source Abbreviation | Accession | Length | Identity | Similarity |
|---------------------|-----------|--------|----------|------------|
| Source Abbreviation | Accession | Length | Identity | Similarity |
| Source Abbreviation | Accession | Length | Identity | Similarity |
47. Tyr-53, and Lys-83 may be the active site residues, making spatially conserved arrangement as seen in human aldose reductase. Ala-123 in PL reductase corresponds to His-117 in human aldose reductase but cannot fill the role of proton donor suggested (10, 11) for His-117 in human aldose reductase. An alternative candidate for this role, Tyr-53, may be involved in the catalytic action of PL reductase. It has been noted that His-117 in human aldose reductase is also less likely to function as the proton donor at physiological pH (21).

Jez et al. (5) have proposed a system for nomenclature of the AKR superfamily based on amino acid sequence identities. They distinguish seven families based on amino acid identities of 40% or more. Since PL reductase shows less than 20% identity with these other AKR proteins, we suggest that it represents a new AKR family. Among several other proteins with a similar amino acid sequence but unknown function, we have cloned and expressed the \textit{iolS} gene of \textit{B. subtilis} in \textit{E. coli} and found that the IolS protein indeed shows PL reductase activity but not aldose reductase activity. Because the \textit{iolS} gene is a structural gene of the \textit{myo-inositol} operon (9), there may be some relation between vitamin B$_6$ and \textit{myo-inositol} metabolism.

Although PL reductase from \textit{S. pombe} showed properties similar to those of the enzyme from \textit{S. cerevisiae} (3), the former enzyme did not catalyze reduction of PLP, an important fact in ascribing a possible function to the enzyme. PLP, a coenzyme form of vitamin B$_6$, is mainly synthesized from PN through the synthetic pathway. However, this may be not the case. As Guirard and Snell (3) have suggested, PL reductase may be involved in salvage of vitamin B$_6$ and excretion of PN from yeast cells by reduction of PL to PN. Actually, we have found that \textit{S. pombe} excretes fairly high amounts of PN into cultivation medium during its cultivation (22). This also supports the possibility that PL reductase play an important role in the excretion of PN from the yeast cell. We are examining the localization of PL reductase.

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REFERENCES
1. Morino, Y., and Sakamoto, Y. (1960) \textit{J. Biochem. (Tokyo) 48}, 733–744
2. Holzer, H., and Schneider, S. (1961) \textit{Biochim. Biophys. Acta 48}, 71–76
3. Guirard, B. M., and Snell, E. E. (1988) \textit{Biofactors 1}, 187–192
4. Molowa, D. T., Shayne, A. G., and Guzelian, P. S. (1986) \textit{J. Biol. Chem. 261}, 12624–12627
5. Jez, J., Flynn, T. G., and Penning, T. M. (1997) \textit{Biochem. Pharmacol. 54}, 639–647
6. Laemmli, U. K. (1970) \textit{Nature 227}, 680–685
7. Sambrook, J., Pritch, E. F., and Maniatis, T. (1989) \textit{Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
8. Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990) \textit{J. Mol. Biol. 215}, 403–410
9. Yoshida, K., Aoyama, D., Ishio, I., Shibayama, T., and Fujita, Y. (1997) \textit{J. Bacteriol. 179}, 4591–4598
10. Wilson, D. K., Bohren, K. M., Gabby, K. H., and Quiocio, P. A. (1992) \textit{Science 257}, 81–84
11. Wilson, D. K., Tarle, I., Petrash, J. M., and Quiocio, P. A. (1993) \textit{Proc. Natl. Acad. Sci. U. S. A. 90}, 9847–9851
12. Rettig, J., Heinemann, S. H., Wunder, F., Lorra, C., Parej, D. N., Dolly, J. O., and Pongs, O. (1994) \textit{Nature 369}, 289–294
13. England, S. K., Ubebe, V. N., Kodali, J., Bennett, P. B., and Tamkun, M. M. (1995) \textit{J. Biol. Chem. 270}, 28331–28334
14. Ireland, L. S., Harrison, D. J., Neal, G. E., and Hayes, J. D. (1998) \textit{Biochem. J. 332}, 21–34
15. Amore, R., Koeter, P., Kuester, C., Cirlacy, M., and Hollenberg, C. P. (1991) \textit{Gene (Amst.) 109}, 89–97
16. Bolen, P. L., Hayman, G. T., and Shepherd, H. S. (1996) \textit{Yeast 12}, 1367–1375
17. Kita, K., Matsuaki, K., Hashimoto, T., Yamanaka, H., Kato, N., Chung, M. C.-M., Kataoka, M., and Shimizu, S. (1996) \textit{Appl. Environ. Microbiol. 62}, 1770–1775
18. Nakamura, K., Kawai, Y., Nakajima, N., and Ohno, A. (1991) \textit{J. Org. Chem. 56}, 4778–4783
19. Nakamura, K., Kondo, S., Kawai, Y., Nakajima, N., and Ohno, A. (1991) \textit{J. Biol. Chem. 266}, 625–636
20. Kahn, A., Van Zyl, C., Van Tender, A., and Prior, B. A. (1995) \textit{Appl. Environ. Microbiol. 61}, 1580–1585
21. Jez, J. M., Bennett, M. J., Schlegel, B. P., Lewis, M., and Penning, T. M. (1997) \textit{Biochem. J. 326}, 625–636
22. Snell, E. E., andowell, B. E. (1971) in \textit{Comprehensive Biochemistry} (Flickin, M., and Sfotz, E. H., eds) Vol. 21, pp. 47–71, Elsevier, Amsterdam
23. Yagi, T., Tanouchi, A., and Hiraoka, Y. (1998) \textit{FEBS Microbiol. Lett. 161}, 1450–150
24. Dayhoff, M. O., Barker, W. C., and Hult, L. T. (1983) \textit{Methods Enzymol. 91}, 524–545

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$3$ T. M. Penning, personal communication.

$4$ M. Ashiuchi, R. Miyake, and T. Yagi, manuscript in preparation.