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Disruption of the *plr1*$^+$ Gene Encoding Pyridoxal Reductase of *Schizosaccharomyces pombe*

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Pyridoxal (PL) reductase encoded by the *plr1*$^+$ gene practically catalyzes the irreversible reduction of PL by NADPH to form pyridoxine (PN). The enzyme has been suggested to be involved in the salvage synthesis of pyridoxal 5′-phosphate (PLP), a coenzyme form of vitamin B_6, or the excretion of PL as PN from yeast cells. In this study, a PL reductase-disrupted (*plr1*Δ) strain was constructed and its phenotype was examined. The *plr1*Δ cells showed almost the same growth curve as that of wild-type cells in YNB and EMM media. In EMM, the *plr1*Δ strain became flocculent at the late stationary phase for an unknown reason. The *plr1*Δ cells showed low but measurable PL reductase activity catalyzed by some other protein(s) than the enzyme encoded by the *plr1*$^+$ gene, which maintained the flow of "PL → PN → PNP → PLP" in the salvage synthesis of PLP. The total vitamin B_6 and pyridoxamine 5′-phosphate contents in the *plr1*Δ cells were significantly lower than those in the wild-type ones. The percentages of the PLP amount as to the other vitamin B_6 compounds were similar in the two cell types. The amount of PL in the culture medium of the disruptant was significantly higher than that in the wild-type. In contrast, PN was much higher in the latter than the former. The *plr1*Δ cells accumulated a 6.1-fold higher amount of PL than the wild-type ones when they were incubated with PL. The results showed that PL reductase encoded by the *plr1*$^+$ gene is involved in the excretion of PL after reducing it to PN, and may not participate in the salvage pathway for PLP synthesis.

Key words: aldo-keto reductase, pyridoxal reductase, *Schizosaccharomyces pombe*, vitamin B_1, vitamin B_6.

Abbreviations: SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; EMM, Edinburgh minimal medium; PL, pyridoxal; PN, pyridoxine; PM, pyridoxamine; PLP, pyridoxal 5′-phosphate; PNP, pyridoxine 5′-phosphate; PMP, pyridoamine 5′-phosphate.

Pyridoxal (PL) reductase [EC 1.1.1.65], which was the first member of eighth family of aldo-keto reductase (*1*), has been purified from a budding yeast, *Saccharomyces cerevisiae* (*2*), and a fission yeast, *Schizosaccharomyces pombe* (*3*). The enzyme catalyzes the reduction of PL by NADPH to yield pyridoxine (PN) and $NADP^+$: the reactivity towards PN is very low and the reaction is practically irreversible under physiological conditions. The enzyme may play an important role in the salvage synthesis of pyridoxal 5′-phosphate (PLP) (Fig. 1): PLP is a coenzyme form of vitamin B_6 and is essential for the catalysis by more than 100 vitamin B_6-dependent enzymes. It has been proposed that PLP is mainly synthesized through the pathway "PL → PN → pyridoxine 5′-phosphate (PNP) → PLP" rather than the pathway " PN → PL → PLP" (*2*). This proposition is based on the reactivities of the enzymes involved in the pathway under physiological conditions. Thus, PL kinase from yeast, unlike those from many other organisms, exhibits substantially higher apparent affinity for PN (K_m, 25 μM) than for PL (400 μM) (*4*), and PL reductase catalyzes the reverse reaction (the oxidation of PN to PL) only at a very limited rate (*3*). In the PLP synthesis pathway proposed, the conversion of PL to PN by PL reductase is essential for the synthesis of PLP. However, the involvement of PL reductase in the synthesis of PLP has not been demonstrated.

Recently, Spenser and coworkers (*5*) demonstrated that 2′-hydroxypyridoxol (2′-hydroxypyridoxine) is a biosynthetic precursor of vitamin B_6 and thiamine (vitamin B_1) in yeast, and predicted that PL is the initial B_6 vitamer formed during *de novo* biosynthesis (Fig. 1, the flow of 2′-hydroxypyridoxol → PL → PN → PNP → PLP). These results suggest that PL reductase plays an important role in the *de novo* synthesis of vitamin B_1 as well as PLP.

S. pombe excretes a fairly high amount of PN into the cultivation medium during its cultivation (*6*) and selectively excretes PN on incubation with excess PL (*7*). The results suggest that the reduction of PL to PN, *i.e.* the PL reductase reaction, is also involved in the excretion of PN. Indeed, the enzymatic properties of PL reductase strongly suggest that the enzyme is involved in PN excretion, as expected previously (*2*, *3*).

Thus, PL reductase may play roles in the syntheses of PLP and thiamine, and the excretion of PN in *S. pombe* cells. It should be examined whether or not a single PL reductase plays these apparently paradoxical roles (synthesis and excretion).

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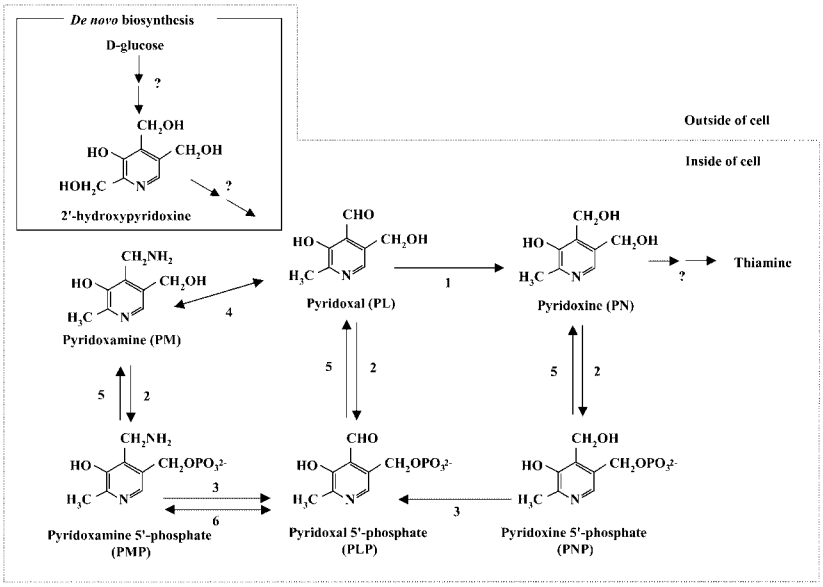

Fig. 1. **Outline of transport and metabolic flow of vitamin B_6 compounds in yeasts.** PL synthesized through the *de novo* pathway (*5*) is reduced to PN by PL reductase. PN is the precursor of thiamine (vitamin B_1) (*26*). The enzymes involved in the PLP synthesis pathway are: 1, PL reductase (PN dehydrogenase, EC 1.1.1.65); 2, PL kinase (2.7.1.35); 3, PMP oxidase (1.4.3.5); 4, assorted apoforms of aminotransferases; 5, assorted phosphatases; 6, assorted aminotransferases.

Here, we have constructed a PL reductase-coding gene (*plr1*$^+$)-disrupted mutant strain of *S. pombe* and characterized its phenotype to elucidate the functional role of the enzyme in the cells. The results show that the PL reductase deleted in this study is actually involved in the excretion of PL after its reduction to PN. It was also found that *S. pombe* cells have other protein(s) with PL reductase activity than PL reductase encoded by the *plr1*$^+$ gene, which is involved in the salvage pathway of PLP synthesis shown in Fig. 1.

EXPERIMENTAL PROCEDURES

Strains and Culture Conditions—Wild-type *S. pombe* strain TP4-5A (*h*$^-$ *leu1 ura4-D18 ade6-M210*) was obtained from Dr. T. Toda (ICRF, UK). A PL reductase-coding gene (*plr1*$^+$)-disrupted strain, which is designated as the *plr1*Δ strain, was derived from *S. pombe* strain TP4-5A. A plasmid vector, pREP41, was transformed by the electroporation method (*8*) into *S. pombe* TP4-5A and *plr1*Δ cells to construct two transformants, TP4-5A/pREP41 and *plr1*Δ/pREP41, respectively. A plasmid, pREP41-*plr1*$^+$, was also introduced into the *plr1*Δ strain to obtain a transformant, *plr1*Δ/pREP41-*plr1*$^+$. The *S. pombe* cells were grown at 30°C with shaking in synthetic medium (YNB medium) containing 1% D-glucose and yeast nitrogen base (*6*), but not amino acids or thiamine, and supplemented with 75 μg/ml each of adenine sulfate, L-leucine, and uracil. Edinburgh minimal medium (EMM) (*9*) containing the supplements was also used.

Plasmid Construction—Plasmid pTPLR1 was constructed by inserting *plr1*$^+$ (1.0-kb) into bacterial expression vector pTrc99A (Pharmacia) as described previously (*3*). Plasmid pT*plr1:: ura4*$^+$ was constructed by inserting the 1.8-kb fragment coding *ura4*$^+$ into the *Pst*I site (reside as 513-bp from 5′ end of *plr1*$^+$) of *plr1*$^+$ in pTPLR1. A 1.8-kb fragment was prepared from plasmid pBura4, which was constructed by inserting *ura4*$^+$ into vector pBluescript II-KS (Toyobo) by PCR, using two oligonucleotides as primers: 5′-GCGCTTAAGCTGCAGTAATGTTGTAGGAGC-3′ and 5′-GCGCTTAAGCTGCAGATATGTATGCATTTGTG-3′, in which the *Pst*I sites are underlined. Plasmid pREP41-*plr1*$^+$ was constructed by inserting *plr1*$^+$ into vector pREP41, which is a *S. pombe* expression vector regulated by the *nmt1* promoter (*10*, *11*) carrying a TATA box mutation (*12*, *13*). *plr1*$^+$ was amplified by PCR using *S. pombe* chromosomal DNA as a template, and two oligonucleotides as primers: 5′-GTTTTCATATGCCTATCGTTAGCGG-3′ and 5′-GTTTTGGATCCTTAAACGGAAAGAGTGCCC-3′, in which the *Nde*I and *Bam*HI sites are underlined, respectively.

Gene Disruption—The *plr1*$^+$ gene was disrupted by inserting *ura4*$^+$ into nearly the middle of its ORF. A 2.8-kb DNA fragment was amplified by PCR using plasmid pT*plr1::ura4*$^+$ as a template, and two oligonucleotides as primers: 5′-GCTCAGAATGATCGACATATACAAC-3′ and 5′-CAAACCAGCTACATAGCACAG-3′. *S. pombe* TP4-5A was transformed with the amplified 2.8-kb fragment by the electroporation method. Positive clones were selected on the synthetic medium not supplemented with uracil, and disruption was verified by PCR using two oligonucleotides as primers: 5′-GTTATAAACATTGGTGTTGGAACAG-3′, which is complementary to the sequence at 500-bp upstream of the 5′ end of *plr1*$^+$ on *S. pombe* genomic DNA, and 5′-GTTCGAATATATTCGGTGCGCC-3′, which is complementary to the sequence at 300-bp downstream of the 3′ end of *plr1*$^+$ on the genomic DNA. The resultant PCR fragment of 3.6-kb confirmed that the disruption cassette was integrated into the *plr1*$^+$ locus.

Immunoblotting—Crude extracts of *S. pombe* cells, prepared by disruption with glass beads as described previously (*14*), were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) by the method of Laemmli (*15*), and then proteins were transferred to polyvinylidene difluoride membranes. Western blot analysis was performed with anti-PL reductase antiserum, which was prepared by immunizing rabbits with 0.1 mg of purified PL reductase mixed with complete Freund adjuvant. The proteins on the membranes were detected by means of chemiluminescence with ECL plus a Western Blotting Detection System (Amersham Phalmacia) (*16*). To eliminate the nonspecific signal, the

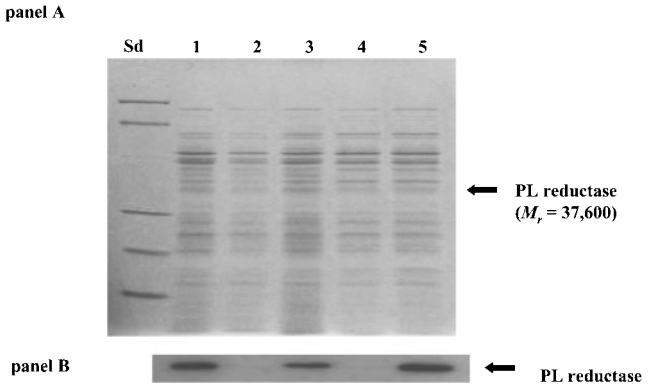

Fig. 2. **Immunodetection of PL reductase in crude extracts of various types of *S. pombe* cells.** Crude extracts (10 μg of protein, each) of *S. pombe* TP4-5A cells (lane 1), *plr1*Δ cells (lane 2), TP4-5A/pREP41 cells (lane 3), *plr1*Δ/pREP41 cells (lane 4), and *plr1*Δ/pREP41-*plr1*$^+$ cells (lane 5), and standard protein mixtures (lane Sd) were loaded on two separate gels and then subjected to SDS-PAGE. One gel was stained with Coomassie Blue (panel A). The other gel was subjected to Western blot analysis with anti-PL reductase antiserum, the stained PL reductase bands being shown in panel B.

anti-PL reductase antiserum was pretreated with a crude extract of *plr1*Δ cells before use.

Analytical Methods—In this study, the natural forms of six vitamin B_6 compounds were identified and measured by reversed-phase isocratic HPLC with fluorescence detection as reported previously (*17*).

Assays—PL reductase was assayed by following the initial decrease in A_{366} of NADPH as described previously (*3*). Samples containing very low levels of enzyme activity were assayed by the HPLC method. The reaction mixture was incubated at 37°C for 2 h and then boiled to stop the reaction, and then the PN produced was measured by the HPLC method. Pyridoxamine 5′-phosphate (PMP) oxidase was assayed colorimetrically with PMP as a substrate (*18*). PL kinase was assayed by determining the amount of produced PLP as described below. The reaction mixture (1.0 ml total volume) contained 4.0 mM PL, which was purified in advance by cation exchange chromatography with Bio-Rad AG 50W-X8, 4.0 mM ATP, 0.1 mM zinc chloride, 0.1 M potassium phosphate (pH 6.4), and crude extract containing 0.1 to 0.5 mg of proteins (*4*). Before use, the crude extract was dialyzed against 200 ml of 0.1 M potassium phosphate (pH 6.4) for 12 h to remove substances of low molecular weight. The reaction mixture was incubated at 37°C for 1 h and then boiled for 3 min to stop the reaction. After centrifugation, the supernatant was applied to a 1 × 1 cm column of Bio-Rad AG 50W-X8, which had been equilibrated with 0.01 M ammonium formate (pH 3.2), to remove the excess PL added as a substrate. The column was washed with 5 ml of the buffer, and then 1 ml of the initial eluate was collected, and PLP in the eluate was measured by the HPLC method. The recovery on this chromatography was more than 95%. Protein was assayed according to the method of Bradford (*19*) with bovine serum albumin as a standard. The active transport activity of PN and PL was assayed as described previously (*7*). The efflux activity of PN was assayed with yeast cells pre-charged with PN as described previously (*20*).

Table 1. **Specific activities of PL reductase, PLkinase, and PMP oxidase in crude extracts of *S. pombe* cells.**

Cells	Wild-type	*plr1*Δ
PL reductase activity[a] (units/mg)	0.54 ± 0.04	0.00044 ± 0.00004[d]
PL kinase activity[b] (units/mg)	0.09 ± 0.01	0.08 ± 0.01
PMP oxidaseactivity[c] (units/mg)	0.27 ± 0.04	0.27 ± 0.02

[a]One unit of PL reductase activity was defined as the amount of enzyme required to reduce 1 μmol of PL per min. [b]One unit of PL kinase activity was defined as the amount of enzyme required to produce 1 nmol of PLP per hour. [c]One unit of PMP oxidase activity was defined as the amount of enzyme required to produce 1 nmol of PLP per min. [d]PL reductase activity measured by HPLC method.

RESULTS

Vitamin B_6 Metabolic Enzyme Activities of the Plr1$^+$ Disrupted Mutant—The PL reductase-coding gene (*plr1*$^+$)-disrupted mutant of *S. pombe*, which was designated as the *plr1*Δ strain, was derived from the *S. pombe* TP4-5A strain by the one step gene replacement method. The *plr1*Δ strain showed only very low (0.08% of the wild type) PL reductase activity (Table 1). Also, a protein band of PL reductase was not detected for the crude extract of *plr1*Δ cells (Fig. 2). Complementation of the *plr1*Δ strain was performed with plasmid pREP41-*plr1*$^+$. The complemented *plr1*Δ/pREP41-*plr1*$^+$ cells showed 2.6-fold higher PL reductase activity than TP4-5A cells, and a dense band on the immunoblotted membrane (Fig. 2, lane 5). Transformation of the wild-type strain with pREP41 caused a 26% loss of PL reductase activity, and the density of the protein band on the immunoblotted membrane was lower than that of the wild-type strain (Fig. 2, lane 3). The transformation of the *plr1*Δ strain with pREP41 did not affect PL reductase activity or the density of its stained band. The residual activity in the crude extract of *plr1*Δ cells was not removed by the antiserum against PL reductase. These results show that the gene disruption was successful and that PL reductase was not expressed in the *plr1*Δ cells. The residual low but measurable PL reductase activity in the *plr1*Δ cells may be catalyzed by an enzyme protein(s) other than PL reductase disrupted here: there are putative genes which show high homology with that of PL reductase, as discussed later. The *plr1*Δ cells showed almost the same PL kinase and PMP oxidase activities as those of the wild-type strain (Table 1). Therefore, the flow of pathway "PN → PNP → PLP" in the synthesis of PLP was not affected by *plr1*$^+$ disruption.

Growth of Plr1Δ Cells—The *plr1*Δ strain could grow in the synthetic medium without vitamin B_6 compounds or thiamine. The growth curve of the *plr1*Δ strain was almost the same as that of the wild-type strain when they were cultured in YNB medium; on cultivation for 24 h they reached the mid-logarithmic growth phase, the absorbance at 600 nm being 1.5, and at 36 h reached the stationary growth phase, the absorbance at 600 nm being 3.0. The results show that PL reductase encoded by the *plr1*$^+$ gene is not necessary for growth of the yeast cells in the synthetic medium.

Table 2. **Intracellular amounts of vitamin B_6 compounds of *S. pombe* wild-type and gene-disrupted cells.**

Cells	Cultivation phase	Intracellular vitamin B_6 (nmol/g, dry cells)					
		PMP	PM	PLP	PL	PN	Total
Wild-type	mid-log	25.58 ± 1.98*	3.36 ± 0.42	1.04 ± 0.32	1.74 ± 0.21	1.11 ± 0.21	32.83 ± 1.67*
		(77.9)	(10.2)	(3.2)	(5.3)	(3.4)	(100%)
*plr1*Δ	mid-log	14.45 ± 0.69	2.88 ± 0.17	0.71 ± 0.42	1.40 ± 0.05	0.55 ± 0.05	19.99 ± 1.37
		(72.3)	(14.4)	(3.6)	(7.0)	(2.7)	(100%)
Wild-type	stationary	15.26 ± 0.11*	2.26 ± 0.17	2.19 ± 0.36	0.81 ± 0.12	0.44 ± 0.07	20.95 ± 0.32*
		(72.8)	(10.8)	(10.5)	(3.8)	(2.1)	(100%)
*plr1*Δ	stationary	11.18 ± 0.98	2.32 ± 0.25	1.69 ± 0.33	0.70 ± 0.09	0.22 ± 0.04	16.11 ± 1.25
		(69.4)	(14.4)	(10.5)	(4.3)	(1.4)	(100%)

Data represent the means of four independent observations and SEM. Values with* are significantly different between the two strains for $p < 0.05$. The figures in parentheses are the amounts of the vitamin B_6 compounds as to the total amount (100%) of vitamin B_6.

Table 3. **Amounts of vitamin B_6 compounds in culture media of the wild-type and *plr1*Δ cells.**

Cells	Cultivation phase	Vitamin B_6 compounds in media (nmol/g, dry cells)					
		PMP	PM	PLP	PL	PN	Total
Wild-type	mid-log	3.47 ± 0.37	3.47 ± 0.11	4.24 ± 0.58	15.37 ± 0.07*	12.93 ± 4.28	39.47 ± 3.94
		(8.8)	(8.8)	(10.7)	(38.9)	(32.7)	(100%)
*plr1*Δ	mid-log	2.35 ± 0.09	2.13 ± 0.22	4.03 ± 0.18	23.77 ± 1.71	5.85 ± 2.77	38.11 ± 4.43
		(6.2)	(5.6)	(10.6)	(62.4)	(15.3)	(100%)
Wild-type	stationary	2.60 ± 0.04	2.71 ± 0.12	10.72 ± 0.64	18.77 ± 0.47*	10.14 ± 0.51	44.93 ± 0.44
		(5.8)	(6.0)	(23.9)	(41.8)	(22.5)	(100%)
*plr1*Δ	stationary	3.03 ± 0.15	2.13 ± 0.02	9.11 ± 0.15	29.45 ± 0.12	UD	43.70 ± 0.10
		(6.9)	(4.9)	(20.8)	(67.4)		(100%)

Data represent the means of four independent observations and SEM. Values with * are significantly different between the two strains for $p < 0.05$. The figures in parentheses are the amounts of the vitamin B_6 compounds as to the total amount (100%) of vitamin B_6. UD means undetectable.

Intracellular Contents of Vitamin B_6 Compounds and Their Amounts in the Culture Medium—To determine the influence of disruption of the *plr1*$^+$ gene on the contents of vitamin B_6 compounds, the intracellular amounts of each vitamin B_6 compound and the total amount in mid-log and stationary phase cells were determined by the HPLC method (Table 2). The total and PMP, which was the most abundant vitamin B_6 compound in the yeast cells, contents in *plr1*Δ cells were significantly lower than those in the wild-type cells. The contents of the other vitamin B_6 compounds were also somewhat lower in *plr1*Δ cells. However, the percentages of PLP as to the other vitamin B_6 compounds in the two cell types were similar: 3.2% and 3.6% of the total vitamin B_6 content, respectively. Although the total content was fairly lower in stationary phase cells compared to that in mid-log cells, similar results as to intracellular contents of vitamin B_6 compounds were obtained with the former cells: lower total and PMP contents in *plr1*Δ cells than in wild-type cells, and the constant PLP percentage in both cell types. The results show that PL reductase encoded by *plr1*$^+$ plays some role by maintaining the PMP content at an appropriate level.

The contents in the culture media were measured, as shown in Table 3. The amounts of PL in the culture media of the *plr1*Δ strain at the mid-log and stationary phases were significantly higher than those in the wild-type strain, although the amounts of PN were lower in the media of the former cells. Interestingly, the PL plus PN content was almost the same in the culture media of the two types of yeast cells, and thus the amounts of total vitamin B_6 were also nearly the same in both cases. The results suggested that PL reductase encoded by the *plr1*$^+$ gene is involved in prevention of PL efflux and/or stimulation of PN efflux from the yeast cells.

Efflux and Uptake Activities of PN—The PN-uptake and efflux activities of the wild-type and *plr1*Δ cells were measured with mid-log phase cells because the uptake activity became very low at the stationary phase, as described previously (*6*). Both types of cells showed similar activities: the activity of the *plr1*Δ and wild-type cells were 20.2 ± 2.9 (nmol/min/g, dry cells) and 17.7 ± 1.0, respectively. PN-efflux activities were measured with cells preloaded with PN as described previously (*20*). The efflux activities of the wild and disruptant cells were almost the same: the activities of the *plr1*Δ and wild-type cells were 152.7 ± 56.3 (nmol/min/g, dry cells) and 167.0 ± 10.4, respectively.

Accumulation of PL and PN on Incubation with PL—The wild-type and disruptant cells were incubated in reaction mixtures containing 10 μM PL, the changes in the contents of vitamin B_6 compounds in the cells being measured (Table 4). The contents of PL and PN, but not those of the other vitamin B_6 compounds, increased in both types of cells. The contents of PN were increased in both cell types. The increase at 1 min-incubation was almost the same for both types of cells, but that at 2 min-incubation was higher for wild-type cells than *plr1*Δ cells. The results show that PL transported into the wild-type and *plr1*Δ cells is easily reduced to PN in conformity with the previous results obtained with another wild-type strain (*6*). Thus, a PL reductase(s) other than that encoded by the *plr1*$^+$ gene sufficiently catalyzes the reduction of PL to PN in the salvage pathway.

Table 4. **Accumulation and formation of various forms of vitamin B_6 on incubation of *S. pombe* cells with PL.**

Strain	Incubation time (min)	Increased intracellular vitamin B_6 (nmol/g dry cells)					
		PN	PL	PM	PNP	PLP	PMP
Wild-type	0	0	0	0	0	0	0
	1	18.46 ± 3.30	4.11 ± 2.24[a]	0	0	0	0
	2	34.82 ± 4.46	6.04 ± 1.82[a]	0	0	0	0
*plr1*Δ	0	0	0	0	0	0	0
	1	17.40 ± 1.76	25.19 ± 4.07[a]	0	0	0	0
	2	25.96 ± 4.56	24.06 ± 1.69[a]	0	0	0	0

Data represent the means of four independent observations and SEM. Values with the same letter in a column (PL, PN) are not significantly different for $p < 0.05$.

In contrast to in wild-type cells, the amount of PL was higher than that of PN in the disruptant cells. Thus, PL reductase encoded by the *plr1*$^+$ gene plays a role by decreasing the content of PL in yeast cells, suggesting that the enzyme stimulates the efflux of PL from the yeast cells after reduction to PN. It is also possible that the enzyme inhibits the transport of PL into the yeast cells. The concentration of PL in the disruptant cells after incubation for 1 min is 51.6 μM if it is assumed that the cells contain 0.488 ml of free intracellular water per g of dry cells (*21*). This result suggests that *S. pombe* cells have an active transport system for PL as well as PN. So far, a transport system for PL has not been found because of its rapid reduction to PN by PL reductase.

Flocculation of Disruptant Cells—When yeast cells were cultured in EMM with the supplements, the wild type and disrupted cells grew at almost the same growth rate while the latter cells became flocculent at the late stationary phase (60 h or later after cultivation) for an unknown reason.

DISCUSSION

The *plr1*Δ strain did not require the supplementation of vitamin B_6 or thiamine for its growth in the synthetic medium, and the intracellular level of PLP, the coenzyme form, was the same as that in the wild-type strain. Thus, PL reductase encoded by the *plr1*$^+$ gene is not necessary for the synthesis of PLP through the salvage pathway. The disrupted cells at the mid-log phase showed 60% and 56% of the total vitamin B_6 and PMP contents, respectively, compared to those in the wild-type cells, suggesting the enzyme is involved in the maintenance of the vitamin B_6 level in yeast cells. The content of PN in the culture medium of disruptant cells was significantly lower than that in the wild-type cells. In contrast, the PL content in the medium of the former cells was much higher than that in the latter cells. When the yeast cells were incubated with PL, the disruptant cells accumulated a much higher concentration of PL than the wild-type cells, although the accumulated PN contents were similar in the two types of cells.

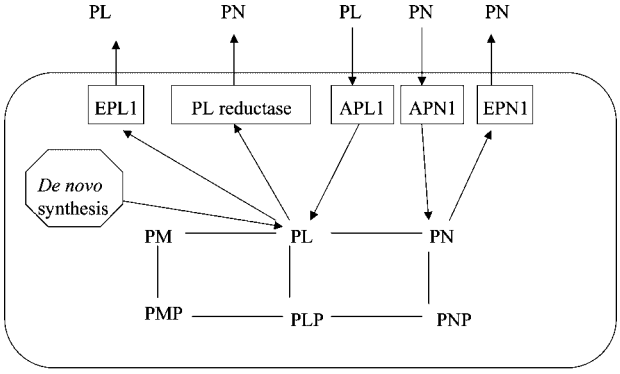

Fig. 3. **A proposal for the function of pyridoxal reductase encoded by the *plr1*$^+$ gene.** EPN1 and EPL1, and APL1 and APN1 denote the assumed efflux proteins of PN and PL, and the assumed active transport proteins of PL and PN, respectively.

These results can be illustrated as shown in Fig. 3. The PL reductase encoded by the *plr1*$^+$ gene could be involved in the excretion of PL after reducing it to PN. The transformation of PL to PN in the salvage pathway may be catalyzed by another protein(s) with PL reductase activity, candidates for which are described below. Yeast cells have an active transport system for PN (*6*) and PL (shown here), in which a transporter like TPN1 (*22*) found recently in the budding yeast may be involved, although a database search showed that *S. pombe* has no gene encoding a TPN1 homologous protein. *S. pombe* cells have an efflux system for PN (*20*), which is shown as EPN1, and may have a passive or active efflux system for PL. The EPN1 system depends on the intracellular concentration of PN, and no PN is excreted when the concentration of PN is lower than 20 μM in the yeast cells (*20*). Because the concentrations of PN in the wild-type and disrupted cells are estimated to be 2.27 μM and 1.1 μM at the mid-log phase, and 0.9 and 0.45 at the stationary phase (based on the date shown in Table 2), the EPN1 efflux system may not practically excrete PN in these cells. Thus, PN in the culture medium may be excreted only by the PL reductase excretion system. Therefore, it is conceivable that the culture medium of the disruptant cells contained no PN at the stationary phase (Table 3), and that the PL concentration in the disruptant cells was much higher than in the wild-type ones when the cells were incubated with PL (Table 4). The finding that the medium at the mid-log phase of *plr1*Δ cells contained 5.85 (nmol/g, dry cells) of PN may indicate that some other efflux system than EPN1 functions before the stationary phase.

Because PL may be the first product of *de novo* synthesis of vitamin B_6 (*5*), it is possible that PLP is directly produced from PL by PL kinase even though its affinity to PL is not high. If this is the case, the production of PN through PL reductase activity is not necessary for PLP

synthesis. Mutant cells with no PL reductase activity are required to estimate the level of direct PLP synthesis from PL. The results of the *S. pombe* genome sequencing project show that the organism contains two homologous PL reductase proteins exhibiting 71% (SPCCI281.04) and 45% (SPAC3A11.11c) identity (*23*). These proteins are the first candidates for other enzymes with PL reductase activity. We are preparing a double (*plr1*$^+$ and each gene encoding a homologous protein) disruptant of *S. pombe*.

The disrupted cells formed flocculates at the late stationary phase, suggesting that PL reductase is directly or indirectly related to the flocculation. The interaction between cell wall sugar-binding protein and mannan (*24*) or galactan (*25*) is important for such flocculation. Thus, the reactivity towards D-mannose and D-galactose of PL reductase was measured: these sugars do not act as substrates for PL reductase (data not shown). Although the natural substrate of PL reductase is PL, the enzyme shows high reactivity towards 2-nitrobenzaldehyde, a synthetic substrate, and it may be possible that PL reductase catalyses the reduction of unknown natural substrate(s), which is intimately involved in the flocculation of *S. pombe* cells. We are studying the mechanism of the PL reductase disruption-dependent flocculation.

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