Efflux System for Pyridoxine in *Schizosaccharomyces pombe*

Kumi HIROSE, Ruamsub CHUMNANTANA, Takashi NAKASHIMA, Makoto ASHIUCHI, and Toshiharu YAGI

Department of Bioresources Science, Faculty of Agriculture, Kochi University, Monobe-Otsu 200, Nankoku Kochi 783-8502, Japan

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Pyridoxine-charged *Schizosaccharomyces pombe* released pyridoxine rapidly at 30°C: very low amounts of three other B₆ vitamers were also released. The rate of efflux was temperature-dependent. The initial rate of efflux was dependent on the concentration of pyridoxine in the cells: the rate was almost zero at lower than 0.02 mM and became saturated at higher than 0.2 mM. Na⁺, sodium azide, and dinitrophenol increased the rate in both the presence and absence of D-glucose. Mg²⁺, thiamine, and menadione inhibited the efflux. The intracellular concentration of ATP did not significantly affect the efflux rate. The system may depend on a membrane potential of the yeast cells. It was found that the fission yeast cells have a gate or carrier system for efflux of pyridoxine, which was distinct from that in *Saccharomyces cerevisiae*.

Key words: efflux; pyridoxine; vitamin B₆; *Schizosaccharomyces pombe*; yeast

Pioneering work on pyridoxine (PN) transport in resting cells of *Saccharomyces carlsbergensis*, a budding yeast, which is now designated as *S. cerevisiae*, has been done by Shane and Snell, who showed an overshoot in intracellular PN concentration when the yeast cells accumulated PN by active transport. It was suggested that intracellular vitamin B₆, or a derivative, activates the exit system of PN, which is involved in the overshoot phenomenon. *S. cerevisiae* also showed the transport overshoot during an uptake of 2-methyl-4-amino-5-hydroxymethylpyrimidine (MAHP) by a thiamine transport system. The efflux system of MAHP was inhibited by 2,4-dinitrophenol, and it was suggested that the yeast cells have an active efflux system for MAHP. Recent studies showed that a fission yeast, *S. pombe*, and a mutant strain of budding yeast accumulate vitamin B₆ compounds such as PN, pyridoxal (PL), pyridoxal 5'-phosphate (PLP), and pyridoxamine 5'-phosphate (PMP) in culture media.

These results suggest that the yeasts have an efflux system for vitamin B₆. However, the mechanism and function of the efflux system have not been studied. We have found that the conversion of accumulated PN to other vitamin B₆ compounds in the fission yeast cells is very slow compared with the budding yeast cells. Thus, *S. pombe* cells containing high amounts of PN after an active transport of PN (PN-charged cells) seemed to be preferable for studying the efflux mechanism of PN.

This paper describes the dependency of an efflux system for PN on the temperature and on intracellular concentrations of PN, and effects of ions, other vitamins, metabolic inhibitors, and concentrations of ATP on the efflux system with the PN-charged cells.

Materials and Methods

*Test organism and growth conditions*. *S. pombe* (IFO 0346) was maintained on a 2.0% (w/v) agar slant containing YES medium. Resting cells of *S. pombe* with a high activity of PN active transport were prepared as described previously.

*Preparation of PN-charged yeast cells*. All manipulations were light-shielded to prevent decomposition of vitamin B₆ compounds. The washed *S. pombe* cells (20 mg, wet weight) were suspended in 10 ml of 20 mM Tris-acetate buffer (pH 4.5) containing 1% (w/v) D-glucose. PN (0.1 mM) was added to the cell suspension and it was incubated at 30°C for 1 h with shaking. After cooling on ice for 5 min, the suspension was centrifuged at 3,500 rpm for 10 min at 4°C, and the cells were washed with 10 ml of cold 1% D-glucose. The PN-charged cells obtained were suspended in cold 1% D-glucose (8 mg/ml) and stored on ice. The PN-charged cells were used within 4 h for an efflux experiment.

*Measurement of efflux and uptake of PN, and con-
centration of intracellular vitamin B₆ compounds.

The cold suspension of PN-charged cells was mixed at once with an equal volume of various warm (60°C) buffers containing 1% D-glucose and various compounds and then the mixture was incubated at 30°C for various times (usually 30 sec). The cell suspensions (1 ml, each) taken at 0 sec (just after the mixing) and 30 sec were filtered separately through a filter unit (DISMIC13 cp, Toyo Roshi, Tokyo) equipped with a cellulose acetate membrane (pore size, 0.2 µm). The filtrates were chromatographed by HPLC to measure vitamin B₆ compounds. Reversed-phase isocratic HPLC with a fluorescence monitor was done as described previously. The subtraction of the value at 0 sec from that of 30 sec gave the amount released.

In the experiment for examination of an effect of D-glucose on the efflux rate, the cold PN-charged cells were washed with cold water just before the experiment.

The intracellular concentration of vitamin B₆ was measured as follows. The cells collected on the filter membrane were washed out with 1.5 ml of cold water, and the cell suspension obtained was boiled for 10 min after a separate measurement of turbidity for estimation of concentration of cells using 100 µl of the suspension. Perchloric acid (9 N, 17 µl) was added to 0.5 ml of the disrupted cell suspension after cooling on ice. After standing on ice for 30 min, the acidic suspension was centrifuged at 12,000 rpm for 10 min. The supernatant was chromatographed by HPLC.

Uptake activity of PN was measured as described previously. The intracellular and extracellular concentrations are expressed as mean ± S.E.M. of four or three independent experiments. The intracellular concentration of PN was calculated assuming fresh cells contain 0.488 ml of free intracellular water per g of dry cells.

Measurement of ATP. The concentration of ATP was measured with a kit (luciferol-LU) purchased from Kikkoman. Photon counting was done with an Aloka luminescence reader BLR-201.

Statistical analysis. A multiple comparison test (the PLSD method of Fisher) was used to compare means.

Results and Discussion

Efflux of vitamin B₆ compounds from PN-charged S. pombe cells

When PN-charged cells were incubated at 0–4°C in 1% D-glucose, only 3.7% of intracellular PN was released after a 60-min incubation: the charged cells could be stored without significant loss of intracellu-

lar PN before starting efflux experiments. On the contrary, PN-charged cells released PN rapidly when they were incubated at 30°C (Fig. 1). The efflux was saturated after 30 min, when 64% of intracellular PN had been released. Because the yeast cells have active transport activity of PN, rates of release and reuptake of PN would be balanced at the saturation level. Low amounts of PMP, pyridoxamine (PM), and pyridoxal (PL) were released. The amounts of PMP, PM, and PL after 90 min of incubation were 22.4 ± 3.0 (nmol/g, wet cells), 16.3 ± 2.5 and 20.6 ± 1.7, respectively. Neither PLP nor pyridoxine 5'-phosphate was detectable. In contrast to the fission yeast cells, the budding yeast cells loaded with ¹⁴C-labeled PN released all six natural B₆ vitamers, among of which the amount of PLP was the highest after 150 min of incubation in Salt M containing 1% D-glucose. Composition of Salt M in grams per liter is: KH₂PO₄ 0.56; (NH₄)₂SO₄ 1.88; KCl 0.42; CaCl₂·2H₂O 0.125; MgSO₄·7H₂O 0.125; FeCl₃·6H₂O 0.0026; and MnSO₄ 0.0026. Thus, the metabolism of PN is strictly controlled in the fission yeast.

This result showed that a short-term efflux should be measured to estimate an accurate efflux rate without the effect of reuptake. Thus, we have measured an initial 30-sec efflux hereafter to measure the efflux rate.

Effects of intracellular concentration of PN on its efflux rate

The efflux rate of PN depended on the concentration of intracellular PN (Fig. 2). The curve is not
strictly hyperbolic but showed dependency of the PN efflux rate on the intracellular PN concentration, and saturation at high concentrations (higher than 0.2 mM). PN was not released when the intracellular concentration of PN was lower than 0.02 mM. Thus, PN may be released through a gate or a carrier, and their function is dependent on the concentration of the intracellular PN. Shane and Snell\(^1\) have also reported that \textit{S. cerevisiae} cells showed very little net efflux at intracellular concentrations of PN less than about 0.02 mM, and the efflux rate became maximum at higher concentrations, although the intracellular concentration of PN at which the efflux rate became maximum has not been identified. The results showed that the fission and budding yeasts have a similar dependency of PN efflux on the intracellular concentration of PN.

\textbf{Effects of glucose, cation, other vitamins, and pH on the efflux}

The initial efflux rates of PN were not affected by D-glucose in the incubation buffers as shown in Fig. 3. On the other hand, Na\(^+\) significantly accelerated the efflux rate of PN. We have shown that Na\(^+\) was required for active transport of PN.\(^3\) Thus, Na\(^+\) is involved in both the efflux and active transport of PN.

Although the effects of Na\(^+\) on the efflux have not been studied with the budding yeast, the effects of D-glucose on the efflux are contradictory: 1% D-glucose increased the efflux rate more than twice with the budding yeast.\(^1\)

Magnesium ion (50 mM) in 20 mM potassium phosphate buffer (pH 4.5) containing 1.0% D-glucose showed different effects on the active transport and efflux rate of PN. The active transport activity was not affected significantly by magnesium ion but the efflux rate was significantly inhibited by magnesium ion: the efflux rates were 8.72 ± 0.73 mmol/g, wet cells and 13.64 ± 1.26 in the presence and absence of magnesium ion, respectively. One possible explanation for the inhibition of the efflux is the known effect of divalent cations on monovalent cation (Na\(^+\)) uptake through the changes produced on the surface potential of the cells.\(^3\)

Interestingly, among the vitamins examined, 50 mM thiamine (vitamin B\(_1\)) and 1 mM menadione (vitamin K\(_1\)) decreased the efflux rate by 71% and 88%, respectively. The effect of thiamine was not abolished by cycloheximide (1 µg/ml), an inhibitor of protein synthesis. We have reported that the active transport of PN was not affected by the same concentration of thiamine.\(^3\) Thus, a carrier or gate of the efflux has a different character from that of the active transport. Thiamine and menadione may interact with the carrier or gate. It is known that the budding yeast requires an external supply of vitamin B\(_6\), when thiamine is present in the culture medium.

The efflux rate of PN was not affected significantly when pH of the incubation buffer (20.0 mM K\(_2\)HPO\(_4\)-20.0 mM K\(_2\)citrate-HCl) was changed from 3.5 to 9.0.

\textbf{Effects of metabolic inhibitors in the presence and absence of D-glucose}

Sodium azide and 2,4-dinitrophenol, which uncouple respiratory chain phosphorylation, significantly increased the efflux rate in both the presence and absence of D-glucose: both compounds had greater effects in the absence of D-glucose (Table I). Ouabaine, a specific inhibitor of Na\(^+\)-K\(^+\) ATPase, and amiloride, an inhibitor of sodium channels, sig-
significantly increased the rate only in the presence of D-glucose. D,L-Ethionine, an inhibitor of ATP pool, and monooiidoacetate, an inhibitor of alcohol fermentation, increased significantly the rate in the absence of D-glucose. The results suggested that the efflux rate of PN may be dependent on ATP levels in the yeast cells and become high when the concentration become low. However, contradictorily, neither FCCP, an uncoupler of respiratory chain phosphorylation, nor p-hydroxymercuribenzoate, an inhibitor of sugar transport, affected the rate. Thus, it could not be concluded that there is a correlation between the efflux rate and the intracellular concentration of ATP based on the effects of inhibitors on the efflux rate.

**Correlation between intracellular concentration of ATP and the efflux rate**

Figure 4 shows a correlation between the intracellular concentration of ATP and the efflux rate of PN. The correlation coefficient was -0.473, showing non-significant correlation between them.

**Effect of various sugars in the absence and presence of inhibitors**

The sugars did not affect the rate in the absence of inhibitors (Table 2). However, in the presence of sodium azide, D-fructose significantly increased the rate: the effect of both the compounds was additive. 2-Deoxy-D-glucose moderately increased the rate. D-Glucose was not effective. Sodium fluoride, an inhibitor of glycolysis, increased the efflux rate 3.6-fold. All the sugars prohibited the action of sodium

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Table 1. Pyridoxine Efflux in the Presence of Various Inhibitors

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Concentration (nmol g⁻¹, wet cells, 30 sec⁻¹)</th>
<th>+ Glucose</th>
<th>− Glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sodium azide</td>
<td>30.0</td>
<td>7.61 ± 1.08</td>
<td>4.37 ± 0.83</td>
</tr>
<tr>
<td>2,4-Dinitrophenol</td>
<td>1.0</td>
<td>43.22 ± 0.51*</td>
<td>56.81 ± 1.60*</td>
</tr>
<tr>
<td>Ouabaine</td>
<td>1.0</td>
<td>17.81 ± 1.88*</td>
<td>34.96 ± 3.80*</td>
</tr>
<tr>
<td>Amiloride</td>
<td>0.5</td>
<td>14.05 ± 0.54*</td>
<td>8.16 ± 0.82</td>
</tr>
<tr>
<td>D,L-Ethionine</td>
<td>0.1</td>
<td>12.61 ± 2.98*</td>
<td>6.66 ± 1.63</td>
</tr>
<tr>
<td>Monooiidoacetate</td>
<td>1.0</td>
<td>9.80 ± 2.79</td>
<td>21.12 ± 9.74*</td>
</tr>
<tr>
<td>p-Hydroxymercuri-benzoate</td>
<td>0.1</td>
<td>8.78 ± 0.86</td>
<td>16.82 ± 2.99*</td>
</tr>
<tr>
<td>FCCP</td>
<td>0.01</td>
<td>7.55 ± 0.76</td>
<td>6.79 ± 3.11</td>
</tr>
</tbody>
</table>

Data represented the means of three independent observations and S.E.M. Values with * are significantly different from the control for p<0.05.

Table 2. Effects of Sugars in the Presence and Absence of Inhibitors

<table>
<thead>
<tr>
<th>Sugars (57 nmol)</th>
<th>Efflux (nmol g⁻¹, wet cells, 30 sec⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>None</td>
</tr>
<tr>
<td>None</td>
<td>15.78 ± 1.41a</td>
</tr>
<tr>
<td>D-Glucose</td>
<td>16.4 ± 2.69a</td>
</tr>
<tr>
<td>D-Fructose</td>
<td>23.35 ± 7.40a</td>
</tr>
<tr>
<td>2-Deoxy-D-glucose</td>
<td>20.50 ± 2.31a</td>
</tr>
</tbody>
</table>

Data represented the means of three independent observations and S.E.M. Values with the same letters in a column are not significantly different for p<0.05.

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Fig. 4. Correlation between Intracellular Concentration of ATP and Efflux Rate of PN. S. pombe cells with different concentrations of ATP were prepared by incubating the cells in different concentrations (0.01% - 1.0%) of D-glucose. Data represent the means of three experiments and S.E.M.
fluoride. The diverse effect of D-fructose and 2-deoxy-D-glucose on the efflux rate in the presence of NaN₃ and NaF could not be interpreted from the viewpoint of a direct correlation of the efflux rate with the intracellular concentration of ATP. This study showed that the metabolism and the efflux system of PN in the fission yeast cells were different from those in the budding yeast cells: the metabolism is strictly controlled and D-glucose did not affect the efflux rate in the former cells. On the other hand, the efflux rate in the two yeast cells showed a similar dependency on the intracellular concentration of PN. It was found that Na⁺ and various metabolite inhibitors increased the efflux rate, and Mg⁺⁺, thiamine, and menadione decreased it. Sugars showed different effects on the inhibition by the metabolic inhibitors. These results suggested that a gate or carrier of PN efflux in the fission yeast cells may be dependent on the membrane potential. The gate or carrier may not be shared with the active transport system even if the systems could interact with each other. Recently, we have suggested that pyridoxal reductase may be involved in the efflux system. We have obtained a preliminary result that the enzyme activity is indeed affected by thiamine and menadione. The study of molecular details of the efflux system is in progress.

References