PnuT uses a facilitated diffusion mechanism for thiamine uptake

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Membrane transporters of the bacterial pyridine nucleotide uptake (Pnu) family mediate the uptake of various B-type vitamins. For example, the PnuT transporters have specificity for vitamin B1 (thiamine). It has been hypothesized that Pnu transporters are facilitators that allow passive transport of the vitamin substrate across the membrane. Metabolic trapping by phosphorylation would then lead to accumulation of the transported substrates in the cytoplasm. However, experimental evidence for such a transport mechanism is lacking. Here, to determine the mechanism of thiamine transport, we purify $PnuT_{Sw}$ from *Shewanella woodyi* and reconstitute it in liposomes to determine substrate binding and transport properties. We show that the electrochemical gradient of thiamine solely determines the direction of transport, consistent with a facilitated diffusion mechanism. Further, $PnuT_{Sw}$ can bind and transport thiamine as well as the thiamine analogues pyrithiamine and oxythiamine, but does not recognize the phosphorylated derivatives thiamine monophosphate and thiamine pyrophosphate as substrates, consistent with a metabolic trapping mechanism. Guided by the crystal structure of the homologous nicotinamide riboside transporter PnuC, we perform mutagenesis experiments, which reveal residues involved in substrate binding and gating. The facilitated diffusion mechanism of transport used by $PnuT_{Sw}$ contrasts sharply with the active transport mechanisms used by other bacterial thiamine transporters.

INTRODUCTION

The ability of enzymes to catalyze complex biochemical reactions is greatly expanded by the use of cofactors, many of which are derived from B-type vitamins. Whereas some bacteria can synthesize these compounds themselves, others lack the necessary anabolic pathways and depend on uptake of the compounds from the environment (Jaehme and Slotboom, 2015a). Uptake (or salvage) is also preferred over de novo synthesis in organisms with complete biosynthetic pathways because it is energetically less costly. Thiamine (vitamin B1) is the precursor of the universal cofactor thiamine pyrophosphate (TPP), which is often involved in decarboxylation reactions (Schellenberger, 1998). Prokaryotic thiamine transport systems are poorly characterized (Jaehme and Slotboom, 2015a), with limited experimental data available only for the ATP-binding cassette (ABC) transporter ThiBPQ (Webb et al., 1998), the energy coupling factor (ECF) transporter ECF-ThiT (Eudes et al., 2008; Rodionov et al., 2009; Erkens and Slotboom, 2010; Erkens et al., 2011; Slotboom, 2014), and very recently the transporter pyridine nucleotide uptake (Pnu) T (Genee et al., 2016).

PnuT belongs to the Pnu-type transporter family (Jaehme and Slotboom, 2015b), which is broadly distributed among bacteria. Besides thiamine transporters, the family also contains transporters for nicotinamide riboside (PnuC) and riboflavin (PnuX, also named RibM), as well as several uncharacterized proteins (Kemmer et al., 2001; Vogl et al., 2007; Jaehme and Slotboom, 2015a,b). PnuC from *Neisseria mucosa* ($PnuC_{Nm}$) is the only Pnu transporter for which a high-resolution structure has been determined (Jaehme et al., 2014). It has a fold that is different from any other transport system, but distantly resembles sugar transporters of the SWEET family. $PnuC_{Nm}$ is a homotrimer, but the monomer appears to be the functional unit. Each protomer of PnuC consists of a conserved core of seven transmembrane helices (TMs; Fig. 1), present in all Pnu members, and an additional N-terminal TM, which is found in only a few members. The nonconserved N-terminal TM is likely needed for trimerization (Jaehme et al., 2014). The conserved core of seven TMs contains two structurally related domains of three TMs, which form a six-helix bundle, and a connecting peripheral TM. The substrate translocation path is predicted to be located in the center of the six-helix bundle (Fig. 1).

Pnu transporters have been hypothesized to work according to a facilitated diffusion mechanism (Kemmer et al., 2001). This hypothesis is based on the operon organization of *pnu* genes, which are often colocalized with kinases that phosphorylate the transported vitamin (Rodionov et al., 2002; Jaehme and Slotboom, 2015b). The co-occurrence of transporter and kinase genes could be indicative of a cytoplasmic metabolic trapping mechanism in which the transported substrates are phosphorylated after transport (Rodionov et al., 2002; Jaehme and Slotboom, 2015a,b).

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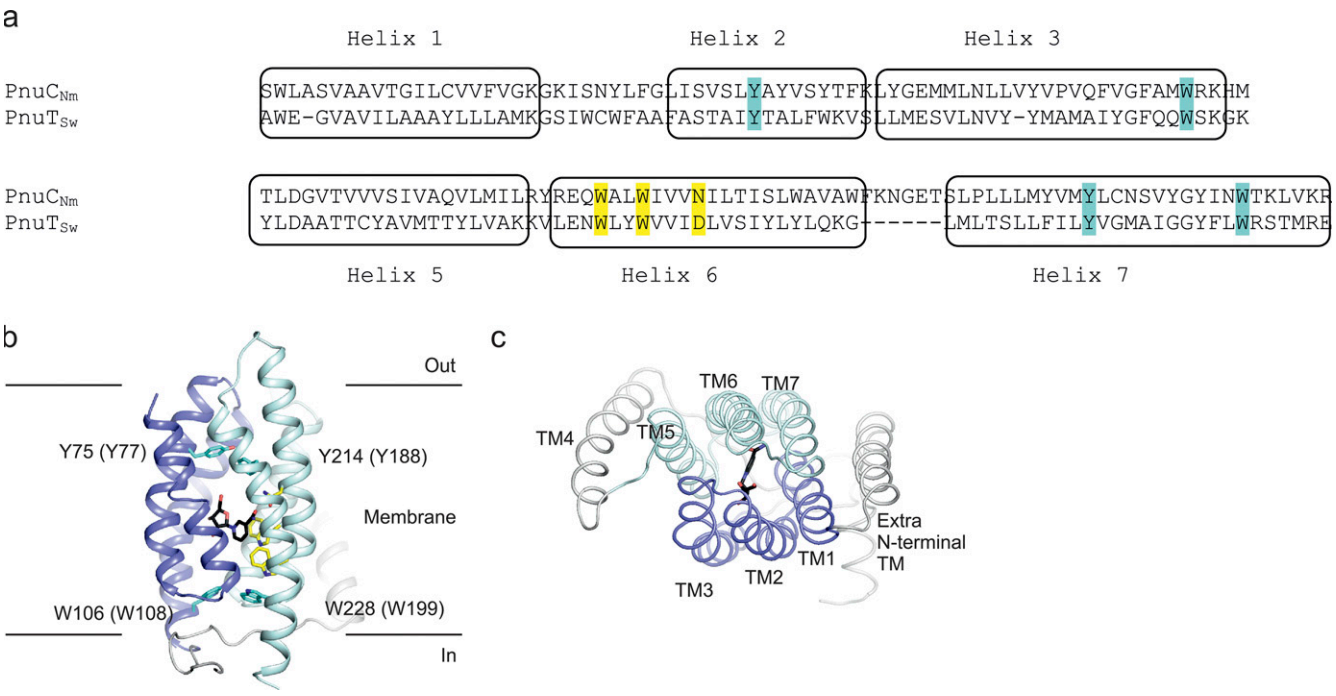

Figure 1. **Structural properties of Pnu transporters. (a)** Sequence alignment of TM1–3 and TM5–7 of PnuC (*N. mucosa*) and PnuT (*S. woodyi*). Conserved residues in the substrate binding site and the potential gate regions are colored yellow and cyan, respectively. **(b)** Substrate binding site of PnuC$_{Nm}$ (Protein Data Bank ID 4QTN) viewed from the membrane plane. A single protomer of PnuC$_{Nm}$ is shown, with the two structurally related three-helix domains TM1–3 and TM5–7 colored blue and cyan, respectively, and TM4 in light gray. The nonconserved N-terminal TM is not shown for clarity. The residues of the binding site and potential gates are colored as in panel (a), and shown in stick representation. Residue numbering according to the sequence of PnuT$_{Sw}$, the numbers in brackets are from PnuC$_{Nm}$. The substrate nicotinamide riboside of PnuC is shown in black stick representation. **(c)** Domain structure of PnuC$_{Nm}$. The protein is viewed from the periplasmic side along an axis perpendicular to the plane of the membrane. The two structurally related three-helix domains TM1–3 and TM5–7 are colored blue and cyan, respectively.

Currently the only experimental evidence for vitamin transport by Pnu transporters comes from in vivo assays (Genee et al., 2016), which usually are not sufficient to establish the mechanism of transport. To determine the mode of transport of PnuT, we produced and purified the PnuT$_{Sw}$ transporter from *Shewanella woodyi*, reconstituted the protein in liposomes, and determined substrate binding and transport properties of the WT protein and mutants. Our data show that PnuT uses a facilitated diffusion mechanism of transport. This mechanism of transport contrasts sharply with the active transport mechanism used by other characterized bacterial thiamine transporters. The study is the first mechanistic characterization of a Pnu transporter.

MATERIALS AND METHODS

Construction of expression plasmids

The gene encoding PnuT from *S. woodyi* was obtained from Life Technologies with a 5′ NcoI restriction site, and a sequence coding for six histidines followed by a stop codon and a HindIII restriction site at the 3′ end of the gene sequence. Additionally, a Gly codon was inserted between the start codon and the second codon of the gene sequence. The sequence was codon-optimized for production in *Escherichia coli*. The described sequence was subcloned via NcoI and HindIII restriction sites into a custom-made p2BAD vector to yield the final expression plasmid (ter Beek et al., 2011; Birkner et al., 2012; Majsnerowska et al., 2015). The PnuT mutants were created by quick-change site-directed mutagenesis.

Protein production

The expression plasmid was transformed into chemically competent *E. coli* MC1061 cells, and the protein was produced as described for PnuC from *N. mucosa* (Jaehme et al., 2014). Cells were cultivated at 37°C, grown until OD_{600} 0.07, and induced with 0.04% L-arabinose. After 2 h of induction at 37°C, cells were collected by centrifugation (20 min, 7,446 *g*, 4°C), washed in wash buffer (50 mM Tris/HCl, pH 8.0), and resuspended in buffer A (50 mM Tris/HCl, pH 8.0, 150 mM NaCl, and 10% glycerol). Cells were lysed by high-pressure disruption (one passage at 25 kPsi at 5°C, Constant Cell Disruption System Ltd.). After cell lysis, 1 mM MgSO4, PMSF, and 50–100 mg/ml DNase were added to the suspension. Cell debris was removed by low-speed centrifugation (20 min, 12,074 *g*, 4°C). Membrane ves-

icles were collected by ultracentrifugation (150 min, 193,727 *g*, 4°C) and resuspended in buffer A to a final volume of 5 ml per liter of cell culture. Subsequently, the membrane vesicles were aliquoted, flash-frozen in liquid nitrogen, and stored at −80°C. The total protein concentration in the membrane vesicles was determined by Bradford Protein Assay (Bio-Rad).

Protein purification

PnuT from *S. woodyi* was isolated as described for PnuC from *N. mucosa* using n-dodecyl-β-D-maltopyranoside (DDM) as detergent for solubilization (1%) and purification (0.05%; Jaehme et al., 2014). Membrane vesicles were thawed rapidly and solubilized in buffer B (50 mM Tris/HCl, pH 8.0, 200 mM NaCl, 15 mM imidazole, 1% [wt/vol] DDM; Anatrace) for 1 h at 4°C, with slow rocking. Unsolubilized material was removed by centrifugation (30 min, 442,907 *g*, 4°C). The supernatant was incubated for 1 h at 4°C with gentle rocking with Ni^{2+} Sepharose resin (column volume of 0.6 ml), which had been equilibrated with buffer G (50 mM Tris/HCl, pH 8.0, 200 mM NaCl, 15 mM imidazole, pH 8.0). Subsequently, the suspension was poured into a 10-ml disposable column (Bio-Rad), and the flow-through was collected. The column material was washed with 20 ml of buffer C (50 mM Tris/HCl, pH 8.0, 200 mM NaCl, 50 mM imidazole, pH 8.0, 0.05% DDM). PnuT protein was eluted in three fractions of buffer D (50 mM Tris/HCl, pH 8.0, 200 mM NaCl, 500 mM imidazole, pH 8.0, 0.05% DDM) of 350, 750, and 650 µl, respectively. 1 mM of EDTA (final concentration) was added to the all-elution fraction to remove coeluted Ni^{2+} ions. Subsequently, the second elution fraction, which contains most of the protein, was further purified by size-exclusion chromatography using a Superdex 200, 10/300 gel filtration column (GE Healthcare), equilibrated with buffer E (50 mM Tris/HCl, pH 8.0, 200 mM NaCl, 0.05% DDM). After size-exclusion chromatography, the fractions containing the protein were combined and used directly for further experiments.

Multiangle laser light scattering coupled to differential refractive-index and UV-absorbance measurements

The oligomeric states of PnuT was determined by size-exclusion chromatography coupled to multiangle laser light scattering and differential refractive-index measurement (SEC-MALLS). SEC-MALLS was performed as described previously (Slotboom et al., 2008; ter Beek et al., 2011). To determine the molecular weight of the protein, the extinction coefficient was calculated with the ExPASy ProtParam tool (Wilkins et al., 1999).

Reconstitution of PnuT

PnuT was reconstituted in liposomes essentially according to the protocol published by Geertsma et al. (2008).

Table 1. **Binding of thiamine and analogues to $PnuT_{Sw}$ determined by isothermal titration calorimetry**

Substrate	Dissociation constant K_d	ΔH	ΔS
	µM	*kJ/mol*	*J/mol/K*
Thiamine	12 ± 5	−27 ± 22	5 ± 71
Pyrithiamine	4.4 ± 1	−18 ± 13	41 ± 43
TMP	No binding		
TPP	No binding		
Oxythiamine	$1.3 * 10^2 \pm 25$	−18 ± 6	14 ± 19

The error represents the SD obtained from three (pyrithiamine, oxythiamine) or five (thiamine) independent measurements.

A lipid mixture consisting of 20 mg/ml *E. coli* polar lipid extract and egg phosphatidylcholine in a ratio of 3:1 (wt/wt) in 50 mM potassium phosphate (KP_i), pH 7.5, was extruded through a 400-nm polycarbonate membrane 11 times and subsequently diluted to 4 mg/ml in 50 mM KP_i, pH 7.5. The preformed liposomes were destabilized by stepwise addition of TritonX-100. The destabilization was followed spectrophotometrically at 540 nm, and TritonX-100 addition was stopped when the value reached two thirds of the initial value. Purified PnuT was mixed with the destabilized lipids in a lipid to protein ratio of 250 (wt/wt). The solution was incubated at 4°C for 30 min before Bio-Beads were added. The Bio-Beads were added in four steps (40 mg/ml per addition), first for 30 min, second for 60 min, third overnight, and fourth for additional 120 min. Afterward, Bio-Beads were removed by filtration, and proteoliposomes were pelleted via ultracentrifugation (286,000 *g*, 25 min, 4°C), resuspended in 50 mM KP_i, pH 7. 5, and stored at 80°C.

Isothermal titration calorimetry (ITC) measurements

For the experiments presented in Fig. 3 and Table 1, ITC experiments were performed using Nano ITC calorimeter (TA Instruments) at the constant temperature of 25°C. ITC cells were loaded with 200 µl of 10–18 µM PnuT in buffer F (50 mM Tris, pH 8.0, 200 mM NaCl, 0.03% DDM). Thiamine (3–5 µM), TMP (thiamine monophosphate; 5 µM), TPP (thiamine pyrophosphate; 5 µM), pyrithiamine (1.5–3 µM), and oxythiamine (5 µM; Sigma-Aldrich) were dissolved in buffer F at indicated concentrations and were titrated into the cell with protein in 1-µl steps. Data were analyzed using software provided by TA instruments.

For the experiments presented in Table 2, ITC measurements were conducted with an ITC200 calorimeter (MicroCal) at 25°C. Thiamine, TMP, TPP, pyrithiamine, and oxythiamine (Sigma-Aldrich) were dissolved in the buffer used for purification at a concentration of 1 mM to 5 mM. The ligand solutions were added in the indicated steps into the temperature-equilibrated ITC cells. The final protein concentration used for ITC measurements was 10–20 µM. Data were analyzed with the ORI GIN-based software (MicroCal).

Table 2. **Binding of thiamine to $PnuT_{Sw}$ mutants determined by isothermal titration calorimetry**

$PnuT_{Sw}$ mutant	Dissociation constant K_d
	μM
Y159F	23 ± 2
Y162F	26 ± 3
Y188A	21 ± 2
Y77A	17 ± 2
D166A	No binding
W199A	12 ± 1
W88A	13 ± 2

The error represents the SD obtained from three independent measurements.

Transport assays

Proteoliposomes were loaded with luminal buffer (50 mM KP_i, pH 7.5) by three cycles of flash-freezing in liquid nitrogen and quick thawing followed by extrusion through polycarbonate membranes with a pore size of 400 nm (11 passages, Avestin). For counter flow experiments, the luminal buffer was supplemented with 1 mM of the indicated ligand. After centrifugation (45 min, 285,775 *g*, 4°C), the proteoliposomes were resuspended in luminal buffer to a final concentration of 0.5 µg PnuT/µl. For each time point in the transport assays, a reaction volume of 100 or 200 µl of outside buffer (50 mM NaP_i, pH 7.5, supplemented with [^{3}H]thiamine [500 nM unless indicated otherwise] and 3 µM valinomycin) was incubated at 25°C while being stirred. For inhibition experiments, thiamine or thiamine analogues were added at a final concentration of 1 mM to the outside buffer. For the assays performed at different values of the membrane potential, a fraction of NaP_i in the outside buffer was replaced by KP_i to set the desired potassium diffusion potential. For the experiments in which the potential sodium ion dependence was tested, the outside buffer contained 1 mM KP_i, pH 7.5, and either 50 mM NaCl or 50 mM choline chloride. Transport was started by adding 1 µg of PnuT, reconstituted in proteoliposomes to the outside buffer. At the indicated time point, 2 ml of stop buffer (ice-cold 50 mM KP_i, pH 7.5) was added, and the reaction was rapidly filtered over a BA-85 nitrocellulose filter. After washing the filter with another 2 ml of stop buffer, the filter was dried for 1 h at 80°C. Subsequently, the filter was dissolved in 5 ml of Filter Count scintillation liquid (PerkinElmer), and the levels of radioactivity were determined using a PerkinElmer Tri-Carb 2800 TR isotope counter.

RESULTS

Oligomeric state of $PnuT_{Sw}$

The only member of the Pnu family of known structure is the nicotinamide riboside transporter $PnuC_{Nm}$, which is a homotrimer. The nonconserved N-terminal TM of the protein constitutes the major part of the oligomerization interface. Many PnuC homologues (such as the proteins from *E. coli* and *Paenibacillus* sp.) lack the N-terminal TM and are monomeric (Jaehme et al., 2014). The PnuT subfamily also lacks the N-terminal helix (Jaehme and Slotboom, 2015b), and we hypothesized accordingly that PnuT proteins are monomeric. We analyzed the oligomeric state of $PnuT_{Sw}$ using static light scattering coupled to size exclusion chromatography (SEC-MALLS) and found that the transporter has a molecular mass of 25 kD in detergent solution, which agrees with a monomeric state (Fig. 2). This observation further supports the hypothesis that the monomer is the functional unit of Pnu transporters, and that the extra N-terminal TM of $PnuC_{Nm}$ is required for oligomerization.

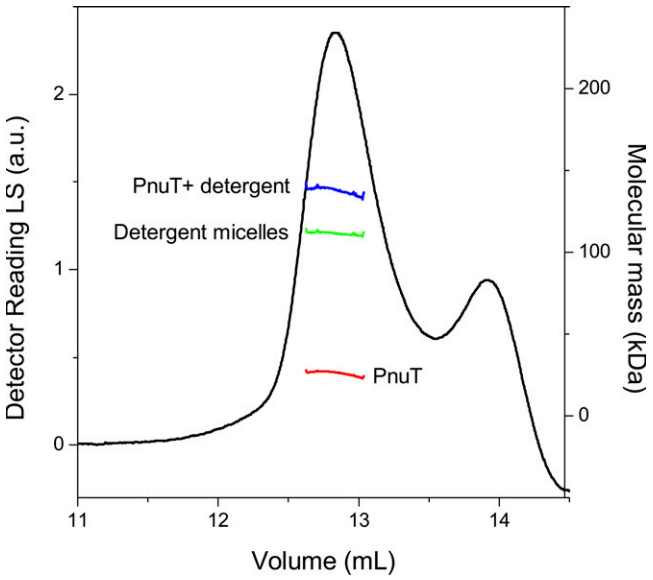

Figure 2. **Static light scattering (SEC-MALLS) analysis of $PnuT_{Sw}$.** The elution profile of a size exclusion chromatography experiment is shown with a black line and calculated molecular mass of protein (25 kD), the detergent micelle (DDM), and protein–detergent complex in red, green, and blue, respectively.

Ligand binding to $PnuT_{Sw}$

We analyzed the ligand binding affinity of purified and detergent-solubilized $PnuT_{Sw}$ by isothermal titration calorimetry (Fig. 3). $PnuT_{Sw}$ binds thiamine and the structural analogue pyrithiamine with affinity constants in the low micromolar range (Fig. 3 a and Table 1, K_d values of 12 µM and 4.4 µM, respectively) and oxythiamine with lower affinity (K_d value of 129 µM). In contrast, binding could not be detected for the phosphorylated thiamine derivatives TMP and TPP. The lack of binding may be caused by the negative charge of the phosphate group or by steric hindrance (Jaehme et al., 2014).

Analysis of the transport mechanism

The transport activity of PnuT transporters has been studied only by whole cell assays (Genee et al., 2016),

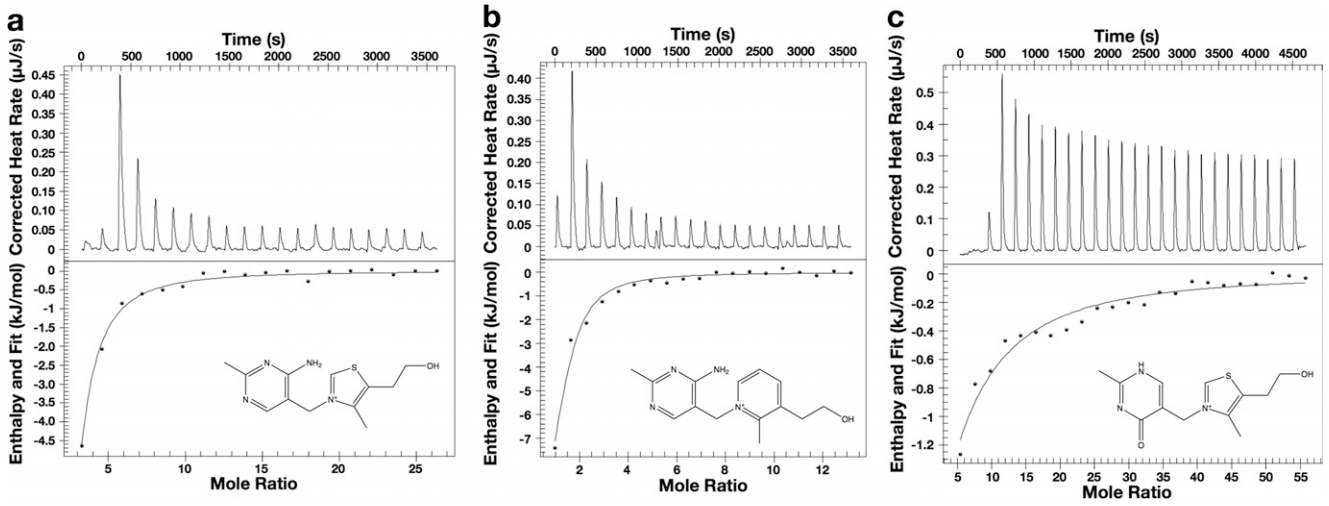

Figure 3. **Substrate binding to PnuT$_{Sw}$. (a–c)** Examples of isothermal titration calorimetry experiments showing (a) thiamine, (b) pyrithiamine, and (c) oxythiamine binding to PnuT$_{Sw}$.

which are not suitable for discrimination between mechanisms using facilitated diffusion and coupled transport. To establish the transport mode, we reconstituted PnuT$_{Sw}$ into proteoliposomes and used a rapid filtration assay to detect transport of tritium-labeled thiamine. The addition of radiolabeled thiamine to the proteoliposomes did not result in detectable transport of the substrate. A possible explanation for the apparent lack of transport is that facilitated diffusion leads to internalization of insufficient amounts of radiolabel for detection. The transport of positively charged thiamine generates a positive-inside membrane potential, which impedes transport of subsequent molecules. In combination with the small internal volume of the liposomes (~14 aL for liposomes extruded through polycarbonate filters with a pore size of 400 nm, which have a diameter of ~300 nm; Geertsma et al., 2008) and the low concentration of radiolabeled substrate used (0.5 µM), the internalized amount radiolabel would not lead to scintillation signals above the background level.

We hypothesized that the accumulation of thiamine inside the lumen of the proteoliposomes could be enhanced by a transmembrane voltage (negative inside). Indeed, the generation of a valinomycin-mediated potassium diffusion potential resulted in robust uptake of thiamine in the proteoliposomes (Fig. 4 a). More negative voltages yielded higher accumulation levels, consistent with the transport of a positively charged substrate. When control liposomes were used that did not have protein reconstituted, voltage-dependent uptake was not observed.

The uptake rates and accumulation level of thiamine were not affected by imposing inward- or outward-directed H^+ gradients, indicating that thiamine transport is not coupled to proton transport (Fig. 4 b). Transport was also not dependent on a sodium ion gradient, as the replacement of Na^+ with choline did not eliminate transport (Fig. 4 c). The lack of proton and Na^+ coupling is consistent with a facilitated diffusion mechanism.

One of the key aspects of facilitated diffusion is that the direction of transport depends solely on the electrochemical substrate gradient. To confirm bidirectionality, we first accumulated thiamine inside the liposomes by applying a membrane voltage, and then added the protonophore carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP), which dissipates the membrane potential (Fig. 4 d). Thiamine was released from the liposomes after addition of FCCP.

Kinetic parameters

We determined the dependence of the initial rates of thiamine transport on the concentration of thiamine to derive values for K_m and V_{max}. The data were fitted to the Michaelis-Menten equation (Fig. 5), yielding values of 14 ± 7 µM for the K_m and 5.0 ± 0.7 pmol thiamine * pmol PnuT^{-1} * min^{-1} for the V_{max}. The V_{max} value suggests that thiamine transport is very slow with only five turnovers per minute, but we argue that the experimentally determined value for the turnover number is likely an underestimation. First, the amount of active protein in the liposomes is difficult to determine. We estimated the amount of protein used for the transport assay from Coomassie staining of reconstituted PnuT, and calculated the turnover numbers based on the—unlikely—assumption that all of the protein used in the reconstitution procedure remained active. Therefore, we likely overestimated the amount of active protein, which leads to an underestimation of the turnover number. Consistently, we did not detect significant binding of radiolabeled thiamine to PnuT to the proteoliposomes, in contrast to what was expected if all protein had remained active. Second, accurate determination

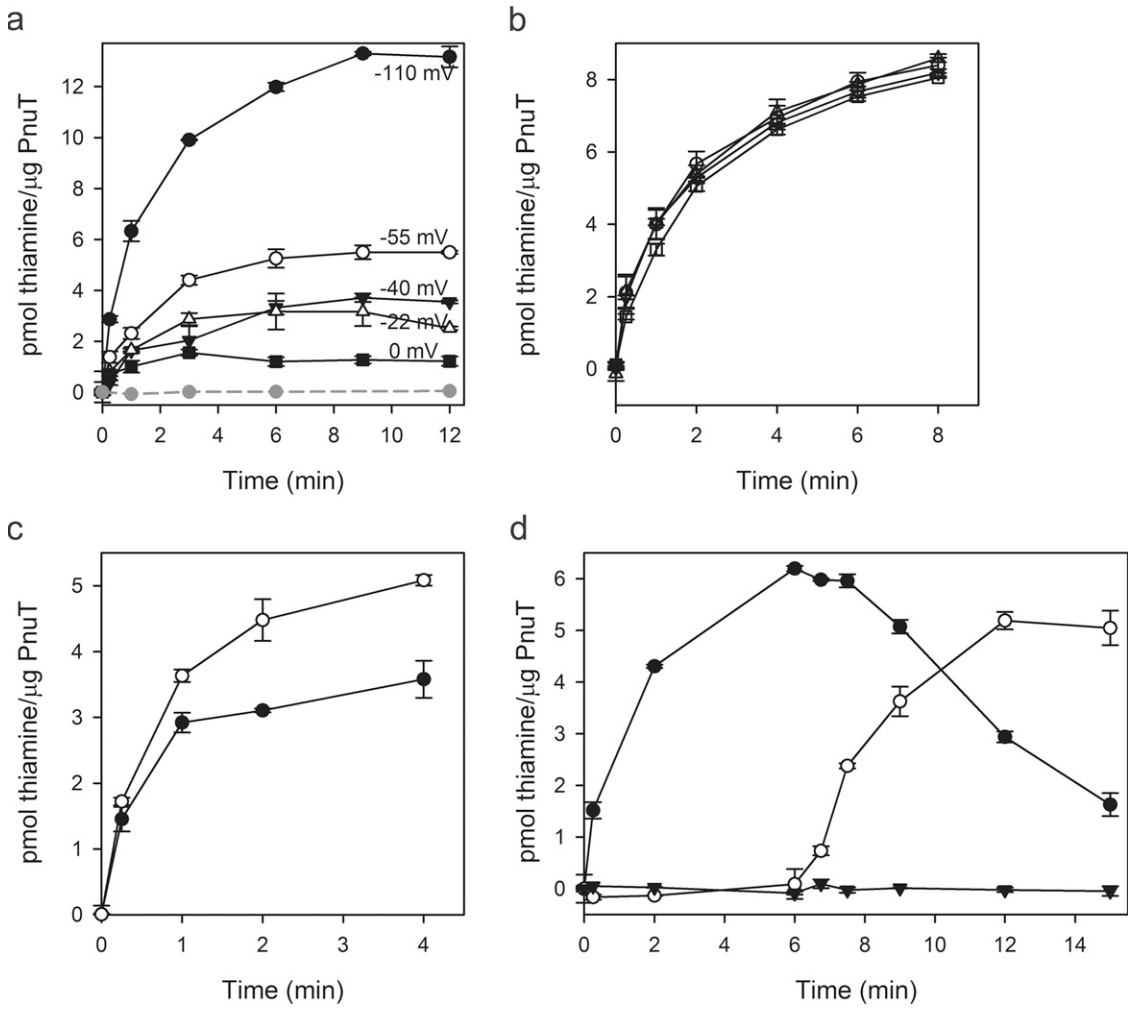

Figure 4. **Thiamine transport by $PnuT_{Sw}$ reconstituted in proteoliposomes. (a)** Transport measured at different voltages across the liposomal membrane: −110 mV (black circles), −55 mV (open circles), −40 mV (black triangles), −22 mV (open triangles), and 0 mV (black squares). The gray dashed line shows the thiamine association to protein-free liposomes (membrane voltage −110 mV). In the latter case, the scale of the y-axis is pmol of thiamine per 250 µg lipids. **(b)** Transport measured at different external pH values: pH 5.5 (squares), pH 6.5 (circles), pH 7.5 (triangles), and pH 8.5 (inverted triangles). The internal pH was 7.5 in all cases. **(c)** Transport measured in the presence (open circles) of absence (black circles) of external Na^+. In the latter case, sodium chloride was replaced by choline chloride. **(d)** Demonstration of bidirectionality of thiamine transport. The luminal and external pH were identical (pH 7.5). Closed circles: The membrane voltage was clamped at −110 mV using valinomycin at the onset of the experiment, and FCCP was added after six minutes. Open circles: Valinomycin was added only after 6 min. Inverted triangles: No valinomycin was present, and FCCP was added after 6 min. All error bars indicate the SD from three experiments.

of initial rates of transport was difficult because of the small internal volume of the liposomes used for these experiments. With multiple proteins present per liposome, only a few turnovers by each of them already leads to a concentration of internalized thiamine close to the thermodynamically achievable accumulation, which leads to underestimation of the maximal rate of transport. Third, the orientation of the reconstituted protein in the liposomes is not known and could have affected the maximal rates of transport.

Inhibition of transport by thiamine analogues

We tested whether the presence of an excess of unlabeled thiamine analogues during the transport assay inhibited the uptake of radiolabeled thiamine into the proteoliposomes (Fig. 6 a). The phosphorylated thiamine derivatives TMP and TTP did not inhibit transport, indicating that the ability of thiamine to interact with the transporter was lost upon phosphorylation of the substrate, consistent with the binding experiments using ITC (Table 1). In contrast, the unphosphorylated thiamine analogues oxythiamine and pyrithiamine blocked transport. This result indicates that the compounds either are transported substrates themselves or are nontransported binders. To discriminate between these possibilities, we performed a series of counter flow assays, in which we first loaded the proteoliposomes with a high concentration of the unlabeled inhibitor, and

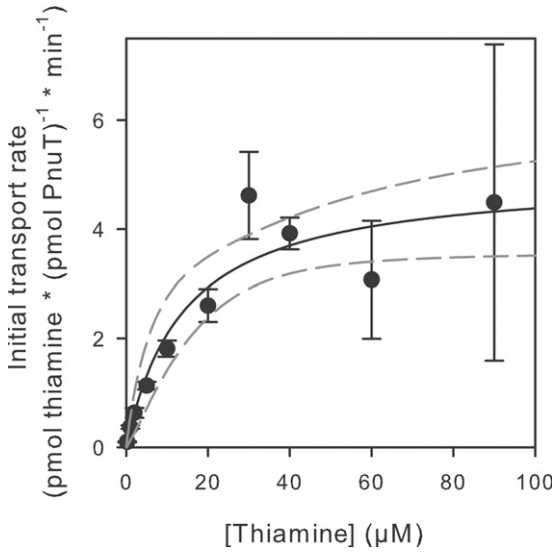

Figure 5. **Determination of K_m and V_{max} of $PnuT_{Sw}$ reconstituted in proteoliposomes for thiamine transport.** The error bars indicate the SD calculated from three independent experiments. The 95% confidence interval is indicated.

then diluted them in a buffer with a low concentration of radiolabeled thiamine (Fig. 6 b). Only transported substrates allow the distribution of the label over the internal and external compartments, which leads to an apparent accumulation of the label in the proteoliposome lumen according to the concentration gradient. TMP or TPP in the proteoliposome lumen did not trigger accumulation of the label, consistent with the lack of inhibition observed. In contrast, both pyrithiamine and oxythiamine led to counter flow of thiamine, indicating that the compounds are transported substrates of PnuT rather than inhibitors that bind only. The lack of transport of phosphorylated forms of thiamine is consistent with the hypothesized facilitated diffusion mode of transport, and metabolic trapping by phosphorylation.

Mutagenesis

$PnuT_{Sw}$ contains all the conserved sequence motifs found in the Pnu transporter family (Fig. 1 a). The crystal structure of $PnuC_{Nm}$ has provided the first clues about the function of the motifs (Jaehme et al., 2014). The $WxxW(x)_3D/N$ motif in TM6 is involved in ligand binding in $PnuC_{Nm}$ (yellow residues in Fig. 1 b). Two tryptophan residues at the C-terminal ends of TM3 and TM7 are hypothesized to form the internal gate (Fig. 1 b; W88, W199, PnuT numbering), and two tyrosines may be part of the external gate (Y77, Y188).

Mutation of the tryptophans (positions 159 and 162) in the $WxxW(x)_3D/N$ motif to alanine destabilized the protein in detergent solution, which prevented purification. In contrast, the mutants W159F and W162F could be purified, and neither of the mutations affected thiamine binding, indicating that the tryptophans are not strictly required for binding. Thiamine binding could not be detected in a mutant of the aspartate from the motif (D166A; Table 2), consistent with a predicted role of the residue in substrate binding in $PnuT_{Sw}$, similar to what was observed in the crystal structure of $PnuC_{Nm}$. Mutation of the proposed gate residues W88, W199, Y77, and Y188 did not affect the binding of thiamine (Table 2). Intriguingly, W88A was unable to transport thiamine, even though thiamine binding was unaffected. This result is consistent with the proposed role of W88 as cytoplasmic gating residue (Fig. 7). For residue W199, which is related to W88 by pseudosymmetry, mutation was less detrimental, with roughly 30% activity remaining for the W199A mutant. Mutation of the proposed periplasmic gating residues Y77 and Y188 had much less effect on activity, suggesting that the periplasmic gating is more complex. Indeed, the crystal structure of PnuC revealed that the periplasmic gate is much "thicker" than the cytoplasmic one, and involves many more residues (Jaehme et al., 2014).

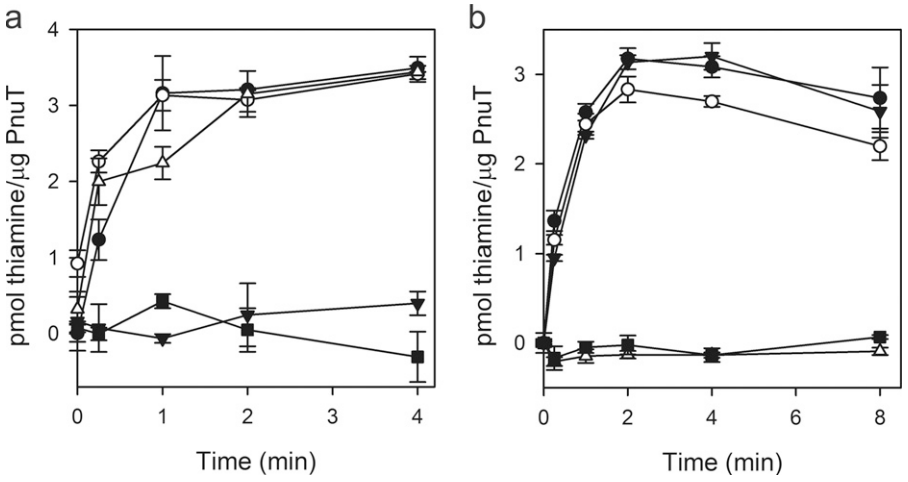

Figure 6. **Competitive inhibition of thiamine transport by $PnuT_{Sw}$ reconstituted in proteoliposomes by thiamine analogues. (a)** [^{3}H]thiamine uptake in the presence of 1 mM of thiamine analogues. TMP (open triangles), TPP (open circles), oxythiamine (black inverted triangles), and pyrithiamine (black squares). Positive control with no additions (black circles). **(b)** Counter flow of [^{3}H]thiamine into proteoliposomes loaded with 1 mM thiamine (black inverted triangles), pyrithiamine (black circles), oxythiamine (open circles), TMP (open triangles), and TPP (black squares). All error bars indicate the SD from three experiments.

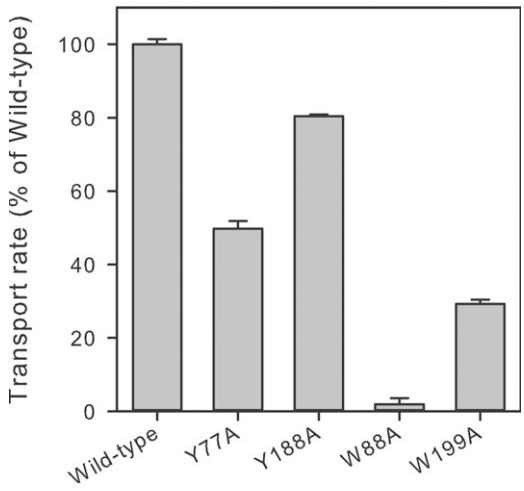

Figure 7. **Initial rates of thiamine transport by $PnuT_{Sw}$ mutants reconstituted in proteoliposomes.** The error is the SD from three experiments.

DISCUSSION

It has been proposed that Pnu transporters operate by a facilitated diffusion mechanism (Mitchell and Moyle, 1958; Boles and Hollenberg, 1997; Vogl et al., 2007), where the transport is solely driven by the electrochemical gradient of the substrate. Our experiments on purified and reconstituted $PnuT_{Sw}$ are consistent with this hypothesis. A negative-inside membrane potential drove the accumulation of the positively charged substrate thiamine, and transport was not coupled to protons or sodium ions. A facilitated diffusion mechanism usually is combined with a mechanism to trap the transported substrate in the cell, which leads to a sustained low cytoplasmic substrate concentration, a maintained inward directed substrate gradient, and the prevention of substrate loss by efflux (Kurnasov et al., 2002). Phosphorylation of thiamine by various thiamine kinases may lead to trapping of compound in the cytoplasm (Kurnasov et al., 2002; Jaehme and Slotboom, 2015b). Such trapping requires that the phosphorylated thiamine derivatives (TMP and TPP; Table 1) are not substrates for $PnuT_{SW}$. Indeed, neither TMP nor TPP could bind to $PnuT_{SW}$ (Fig. 1 c), consistent with the putative trapping mechanism. In contrast, the ECF-type ABC transporter ECF-ThiT, which is found only in organisms lacking PnuT, does accept phosphorylated thiamine analogues as substrates (Erkens and Slotboom, 2010). Such broader substrate specificity may be a selective advantage in some conditions. However, the price paid for the broader substrate specificity is that ATP hydrolysis is required to accumulate the substrate in the cell, which makes the transport essentially unidirectional.

Transporters that work according to a facilitated diffusion mechanism are typically low-affinity, high-capacity transport systems, such as the mammalian glucose transporters of the GLUT family, which exhibit affinities in the mM range (Boles and Hollenberg, 1997). Low-affinity, high capacity transporters are suitable for transport of compounds (such as sugars) present in high concentrations and needed in large quantities. $PnuT_{Sw}$ has a K_m value for thiamine transport of 14 µM (Fig. 5). Compared with the GLUT transporters, $PnuT_{Sw}$ is a high-affinity transport system, but the qualification "high-affinity" is relative. Compared with other thiamine transport systems, which are found in different organisms, the affinity of $PnuT_{Sw}$ is low. The ECF-type ABC transporter ECF-ThiT has a binding affinity for thiamine that is five orders of magnitude higher than that of $PnuT_{Sw}$ (K_d ∼0.1 nM; Erkens and Slotboom, 2010; Swier et al., 2015; Monjas et al., 2016). Similarly, the ABC transporter ThiBPQ binds thiamine with nanomolar affinity (Kawasaki et al., 1969). The ECF and ABC transporters bind thiamine to their membrane-embedded S-components and periplasmic binding proteins, respectively, followed by transport into the cytoplasm. Expression of large amounts of the high-affinity binding proteins allows for scavenging of the limited amounts of substrates from the environment, which may yield a selective advantage (Rodionov et al., 2009; Erkens et al., 2011). The high-affinity binding of thiamine to the ABC and ECF transporters comes at a price, because ATP hydrolysis is needed to release the substrate in the cytoplasm at useful rates (Xu et al., 2013; Swier et al., 2016). In contrast, the lower-affinity–facilitated diffusion mechanism used by PnuT does not require hydrolysis of ATP, and therefore is energetically cheaper.

S. woodyi, as well as several other marine organisms of the γ-proteobacteria that contain PnuT proteins (*Alteromonas*, *Shewanella*, or *Glaciecola*), are found in a marine environment with typically low thiamine concentrations (picomolar range; Sañudo-Wilhelmy et al., 2012; Jaehme and Slotboom, 2015b). It is therefore questionable whether PnuT proteins are primarily used for uptake of thiamine. As *S. woodyi* appears to encode the complete metabolic pathway for synthesis of thiamine, it is possible that the organism uses PnuT for the release of surplus unphosphorylated thiamine. Release of vitamins by facilitators from the Pnu family may be of use to engineer vitamin production strains that secrete increased amounts of the compounds. This idea has been tested for the production of riboflavin, using the riboflavin-specific Pnu transporter PnuX form *Corynebacterium glutamicum* (Vogl et al., 2007). PnuX showed similar substrate affinity as $PnuT_{Sw}$ with a reported K_m value of 11 µM, determined in whole cells (Vogl et al., 2007). The value indicates that the micromolar affinity may be a general property of Pnu transporters rather than a peculiarity of $PnuT_{Sw}$. The similarity in structure, sequence, and genomic colocalization with specific kinases between PnuT and Pnu transporters of different substrate specificity suggests

that the transport mechanism might apply more generally to the Pnu transporter family.

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