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Biochemical Characterization of ThiT from *Lactococcus lactis*: A Thiamin Transporter with Picomolar Substrate Binding Affinity[†]

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ABSTRACT: The putative thiamin transporter ThiT from *Lactococcus lactis* was overproduced in the membrane of lactococcal cells. *In vivo* transport assays using radiolabeled thiamin demonstrated that ThiT indeed was involved in thiamin transport. The protein was solubilized from the membranes and purified in detergent solution. Size exclusion chromatography coupled to static light scattering, refractive index, and UV absorbance measurements (SEC-MALLS) showed that ThiT is a monomer of 22.7 kDa in detergent solution. When the cells overexpressing ThiT had been cultivated in complex growth medium, all binding sites of the purified protein were occupied with substrate, which had copurified with the protein. MALDI-TOF mass spectrometry analysis confirmed that the copurified substance was thiamin. Substrate-depleted ThiT was obtained by expressing the protein in cells that were cultivated in chemically defined growth medium without thiamin. The intrinsic tryptophan fluorescence of substrate-depleted ThiT was strongly quenched upon thiamin binding. The quenching of the fluorescence was used to determine dissociation constants for thiamin and related compounds. ThiT had an unusually high affinity for thiamin ($K_D = 122 \pm 13$ pM) and bound the substrate with a 1:1 (protein:ligand) stoichiometry. TPP, TMP, and pyrithiamin bound to ThiT with nanomolar affinity. A multiple sequence alignment of ThiT homologues revealed that well-conserved residues were clustered in a tryptophan-rich stretch comprising the loop between the predicted membrane spanning segments 5 and 6. Mutational analysis of the conserved residues in this region combined with binding assays of thiamin and related compounds was used to build a model of the high-affinity binding site. The model was compared with thiamin binding sites of other proteins and interpreted in terms of the transport mechanism.

Thiamin (vitamin B_1) is the precursor of TPP,[1] an important cofactor for a wide variety of enzymes that catalyze decarboxylation reactions (*1*, *2*). The TPP utilizing enzymes are involved in many essential cellular processes such as the citric acid cycle and the pentose phosphate pathway, common to organisms in all kingdoms of life.

Many bacteria are capable of thiamin synthesis via diverse biosynthetic pathways (*3*, *4*). In addition, genes coding for putative or confirmed transport systems for the uptake of thiamin have been found in many prokaryotic genomes (*5*), but the proteins catalyzing bacterial vitamin transport are poorly characterized. The best studied example is the ABC (ATP binding cassette) transporter ThiBPQ from *Salmonella typhimurium* (*6*) that mediates thiamin and TPP transport at the expense of ATP hydrolysis. Binding of thiamin and thiamin phosphates is carried out by the ThiB (also named TbpA), a soluble substrate binding protein (SBP) that resides in the periplasmic space (*7*, *8*). ThiB delivers its substrate to the membrane-bound ThiP, and transport is driven by ATP binding and hydrolysis by the ThiQ subunit in the cytosol.

Recently, we have identified a different type of prokaryotic thiamin transporter: ThiT from *Lactobacillus casei* (*9*). ThiT is a member of the energy coupling factor (ECF) transporters, a new class of transport proteins that shares some resemblance with ABC transporters. ECF transporters consist of a conserved tripartite complex with two identical or homologous nucleotide binding proteins/domains (EcfA and EcfA′) and a small (~30 kDa) integral membrane protein EcfT (*10*). In contrast to ordinary bacterial ABC transporters for substrate uptake, ECF transporters do not employ soluble SBPs but instead use small (~20 kDa) integral membrane proteins with five to six predicted transmembrane helices for substrate recognition and binding. These proteins are termed the core transporters, and ThiT is an example. Besides ThiT, a plethora of other (putative) core transporters have been found encoded in the genomes of prokaryotes, each specific for a different substrate. Their expression is often under the control of a riboswitch (*5*, *11*, *12*) and thus regulated by the intracellular concentration of their substrates. Core transporters can interact with the tripartite ECF protein complexes, forming a complete transport system likely to resemble the basic architecture of an ABC transporter, consisting of two transmembrane domains and two nucleotide binding domains. Surprisingly, there are indications that the core transporter alone can also mediate transport, albeit not coupled to ATP hydrolysis but most likely via a secondary transport mechanism (*13*).

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[1]Abbreviations: MALDI, matrix-assisted laser desorption ionization; TOF, time of flight; CDM, chemically defined medium; TPP, thiamin pyrophosphate; TMP, thiamin monophosphate; SBP, substrate binding protein; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; LC, liquid chromatography; DDM, *n*-dodecyl β-D-maltopyranoside; DM, *n*-decyl β-D-maltopyranoside; OD_{600}, optical density at 600 nm; IMAC, immobilized metal affinity chromatography; PSI-BLAST, position-specific iterated BLAST.

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Here we present a biochemical analysis of the ThiT homologue llmg_0334 from *L. lactis*, and we evaluate the interactions responsible for high-affinity substrate recognition. A detailed model of the thiamin binding site is valuable for the elucidation of the transport mechanism of the ECF membrane transporters. In addition, it could provide a framework for antimicrobial drug design, as studies on the pathogen *Listeria monocytogens* (*14*) showed that the Gram-positive bacterium is dependent on ThiT for its intracellular replication, because it lacks a complete thiamin biosynthesis pathway. When the *thiT* gene (lmo1429) was knocked out, growth inside Caco-2 cells was severely diminished compared to that of a control strain effective in thiamin transport.

EXPERIMENTAL PROCEDURES

Materials. Thiamin, TMP, TPP, pyrithiamin, 4-aminopyrimidine, 2-methylpyrimidine, and 4-methyl-5-thiazoleethanol were obtained from Sigma. Ni-Sepharose was from GE Healthcare, detergents were from Anatrace, and [^{3}H]thiamin was obtained from American Radiolabeled Chemicals. All chemicals were of analytical grade.

Construction of Plasmids. The gene coding for ThiT was amplified with PCR from *L. lactis* MG1363 chromosomal DNA with primers containing 5′ *Pci*I and 3′ *Spe*I restriction sites (for primer sequences, see Supporting Information). The PCR products were digested with *Pci*I/*Spe*I and ligated in the vector pREnHis (see Supporting Information) containing the sequence coding for an N-terminal His_8 tag, yielding the vector pREnHis-ThiT. Site-directed mutagenesis of ThiT was performed using the megaprimer approach (*15*, *16*) followed by ligation of the mutated ThiT gene in pREnHis. All cloned fragments were verified by DNA sequencing (ServiceXS, The Netherlands). Finally, the pREnHis constructs were converted to *L. lactis* expression vectors using the vector backbone exchange (VBEx) protocol (*17*).

Overexpression of ThiT and ThiT Mutants. *L. lactis* strain NZ9000 (*18*) was used for nisin induced overexpression. Cells were grown semianaerobically in chemically defined medium (*19*) or M17 broth (Difco) supplemented with 2.0% (w/v) glucose and 5 μg/mL choramphenicol in a 2 L bioreactor at pH 6.5 and 30 °C. To produce substrate-free ThiT, CDM was prepared from which thiamin was omitted. ThiT expression was induced at an OD_{600} of 1.5 by the addition of 0.1% (v/v) culture supernatant from the Nisin A producing strain NZ9700 (*18*). The cells were allowed to continue growing for ~3 h and harvested at a final OD_{600} of 5−6. After centrifugation for 15 min at 5000*g*, the cells were resuspended in 50 mM potassium phosphate (KP_i), pH 7.0, frozen in liquid nitrogen, and stored at −80 °C.

Preparation of Membrane Vesicles. Membrane vesicles were prepared by lysis with a high-pressure cell disruptor (Constant Cell Disruption Systems), using two passages at 39000 psi and 4 °C. Prior to the disruption, $MgSO_4$ (5 mM) and DNase (100 μg/mL) were added. Cell debris was separated from the membrane vesicles by a low-speed spin at 18500*g* for 15 min at 4 °C, and the membrane vesicles were pelleted by centrifugation at 150000*g* for 1.5 h at 4 °C. The membranes were resuspended in 50 mM KP_i, pH 7.0 (final concentration ~10 mg/mL), frozen in liquid nitrogen, and stored at −80 °C.

Purification of ThiT-His. Membrane vesicles were rapidly thawed and resuspended to a concentration of ~5−8 mg/mL in buffer A (50 mM KP_i, pH 8.0, 200 mM KCl, and 10% (v/v) glycerol). The vesicles were solubilized with 1% (w/v) DDM for 45 min on ice. Unsolubilized material was removed by centrifugation at 270000*g* for 15 min at 4 °C. The supernatant was incubated with nickel-Sepharose (500 μL bed volume) for 45 min at 4 °C while gently rotating. Next, the suspension was poured into a 10 mL disposable column (Bio-Rad), and the flow-through was discarded. The column was washed with 16 column volumes (CV) of buffer A, supplemented with 40 mM imidazole and 0.05% (w/v) DDM and 16 CV of buffer B (50 mM KP_i, pH 8.0, 200 mM KCl) supplemented with 30 mM imidazole and 0.05% (w/v) DDM. ThiT was eluted in three fractions of one CV with buffer B supplemented with 500 mM imidazole and 0.05% (w/v) DDM. The second elution fraction (containing most of the purified protein) was loaded on a Superdex 200 gel filtration column (GE Healthcare) equilibrated with buffer C (50 mM KP_i, pH 7.0, 150 mM KCl, and 0.05% (w/v) DDM). Fractions containing ThiT were collected and used directly for further analysis.

MALDI-TOF Mass Spectrometry and Identification of ThiT-Bound Substrate. To extract the bound ligand, purified ThiT-His (~1 nmol of protein) was diluted to 20 μL in 0.1% trifluoroacetic acid; 0.2% SDS was added to denature the protein and release the substrate. Desalting and protein removal were accomplished by ZipTip purification (Millipore). The resulting extract was further purified using nano-LC on a C18 reversed-phase column. From each LC fraction, a mass spectrum was recorded on a MALDI-TOF/TOF instrument (4700 proteomics analyzer; Applied Biosystems) with α-cyano-4-hydroxycinnamic acid as a matrix. Serial dilutions of a thiamin−HCl solution were directly spotted on the MALDI plate to generate reference spectra for thiamin.

Bioinformatics. Conserved residues in ThiT were found with a PSI-BLAST, followed by a multiple sequence alignment as performed by the online tool FRpred (*20*). Membrane topology predictions were made with TOPCONS (*20*−*24*).

Static Light Scattering and Refractive Index Measurements To Determine the Oligomeric State. Determination of the molecular weight of purified ThiT was performed as described (*25*). For the purification of ThiT in DM, DDM was replaced by 0.15% (w/v) DM in all steps, except for the solubilization.

Substrate Binding Measurements by Fluorescence Titration. Fluorescence was measured on a Spex Fluorlog 322 fluorescence spectrophotometer (Jobin Yvon) in a 1000 μL stirred quartz cuvette at 25.0 °C. Purified ThiT was diluted in buffer C to a concentration of 15−50 nM (final volume 800 μL) and incubated for 5 min to reach temperature equilibrium. The substrates were added in 0.5−2.0 μL steps using a syringe pump (Harvard apparatus) fitted with a 500 μL gastight glass syringe (Hamilton Co.). The syringe was connected to the cuvette by tubing with an internal diameter of 0.13 mm (Vici AG International). The excitation wavelength was 280 nm, and emission was measured at 350 nm. The signals were averaged over a period of 20 s. After each substrate addition, a 5 s interval was allowed for equilibration and mixing.

Data Analysis. The change in tryptophan fluorescence after each addition of substrate was calculated according to eq 1:

$$\Delta F_i = \left(\frac{V_0}{V_0 + d_i}\right) \cdot F_0 - F_i \qquad (1)$$

in which V_0 is the sample volume at the start of the titration, d_i is the volume of titrant added after the ith addition, F_0 is the

average fluorescence at the start of the titration, and F_i and ΔF_i are the average fluorescence and fluorescence quenching after the ith substrate addition. The obtained values for ΔF_i were plotted as a function of the substrate concentration. The resulting curve was fitted to eq 2 in Origin 7.0 (OriginLab) to obtain a value for the K_D and n:

$$\Delta F = A\left(\frac{n+[S]+K_D}{2} - \frac{\sqrt{(n+[S]+K_D)^2 - 4n[S]}}{2}\right) \quad (2)$$

where A is the proportionality factor, n is the concentration of binding sites in the cuvette, K_D is the dissociation constant, and [S] is the concentration of substrate in the cuvette. Dilution of the substrate and protein concentration was corrected by inserting eq 3 and 4 in eq 2:

$$n = n_0\left(\frac{V_0}{V_0 + d_i}\right) \quad (3)$$

$$[S] = [S]'\left(\frac{V_0}{V_0 + d_i}\right) \quad (4)$$

with $[S]'$ being the concentration of substrate uncorrected for dilution and n_0 the concentration of binding sites at the start of the titration.

For medium- to low-affinity binding (K_D > 10× protein concentration), the values were fitted to eq 5, describing equilibrium binding with a single binding site:

$$\Delta F = \frac{B[S]}{K_D + [S]} \quad (5)$$

where B is the maximum quenching, measured at saturating substrate concentrations.

In Vivo Thiamin Transport Assay. Twenty-five ml of CDM (supplemented either with 5 μM thiamin or without thiamin) was inoculated with 2% (v/v) of an overnight culture (in M17 medium) of *L. lactis* NZ9000 carrying either the empty plasmid pNZ8048 or pNZnHis-ThiT. At an OD_{600} of 0.6−0.7, nisin was added as described above and growth was allowed for an additional hour, yielding a final OD_{600} of ~1. The cells were washed twice with ice-cold 50 mM K^+-HEPES pH 7.0, resuspended in the same buffer to an OD_{600} of 20 and kept on ice until further use.

For the transport assay, the cells were diluted to an OD_{600} of 10 in 50 mM K^+-HEPES pH 7.0 supplemented with 10 mM glucose and energized for 5 min at 30 °C. The uptake reaction was started by the addition of 31.5 nM ^{3}[H]-thiamin together with unlabeled thiamin (total thiamin concentration 1 μM). At the indicated time points, 150 μL samples were taken and diluted in 2 mL ice-cold 50 mM K^+-HEPES pH 7.0, followed by rapid filtration trough a 0.45 μm pore-size cellulose nitrate filter. The filters were washed twice with 2 mL 50 mM K^+-HEPES pH 7.0 and dried at 80 °C for 1 h. Two ml scinitilation liquid (emulsifier scintillator plus, Perkin-Elmer) was added to dissolve the filters and the radioactivity was determined with a Tri-Carb 2800TR liquid scintillation analyzer (Perkin-Elmer).

RESULTS

Identification of llmg_0334 as the Gene Encoding the L. lactis Thiamin Transporter ThiT. The *thiT* gene (llmg_0334) on the *L. lactis* MG1363 genome is preceded by a predicted TPP riboswitch (*5*, *11*). TPP riboswitches act as negative regulators of expression, preventing translation when the intracellular concentration of TPP is high, but allowing translation of the mRNA when TPP concentrations are low (*11*, *26*). This pattern of regulation was reflected by *in vivo* thiamin uptake assays in wild-type *L. lactis* cells (Figure 1). When thiamin was plentiful in the growth medium, no significant uptake of thiamin was measured because the production of endogenous ThiT was repressed. In contrast, cells cultivated in the absence of thiamin derepressed the translation of ThiT and readily transported [^{3}H]thiamin. When *thiT* was overexpressed from a plasmid without the riboswitch sequence and under the control of the *nisin A* promoter, significant uptake of [^{3}H]thiamin was observed regardless of the thiamin concentration in the medium, showing the direct involvement of ThiT in thiamin transport. Cells overproducing recombinant ThiT showed very similar [^{3}H]Thiamin transport rates as wild-type cells expressing only endogenous *thiT*. We tentatively conclude from this result that the levels of the endogenous ECF tripartite complex could be rate limiting for thiamin uptake. These levels are most likely not affected by the concentration of thiamin in the medium nor by the overexpression of the *thiT* gene and thus result in similar transport rates for wild-type and overexpressing cells. In contrast, the *binding* of [^{3}H]thiamin to the cells increased upon overexpression of ThiT (offset at the y axis), indicating that isolated ThiT may be primarily involved in binding or slow turnover transport.

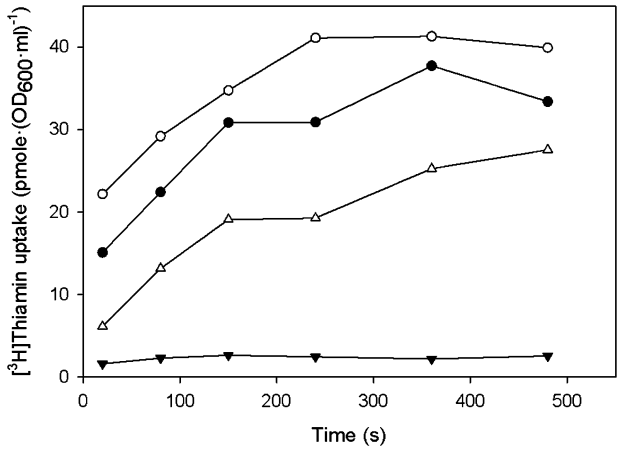

FIGURE 1: Uptake of [^{3}H]thiamin in *L. lactis* cells. Key: open circles, cells expressing ThiT-nHis grown in CDM without thiamin; closed circles, cells expressing ThiT-nHis in medium supplemented with thiamin; open triangles, control strain harboring an empty plasmid, grown in medium without thiamin; closed triangles, the control strain grown in thiamin supplemented medium.

Purification of ThiT and Determination of the Oligomeric State. His-tagged ThiT (His_8-ThiT) was overproduced in *L. lactis* membranes, solubilized with the detergent DDM, and purified using Ni^{2+}-affinity and size exclusion chromatography (Figure 2). From the yield of purified ThiT, it was estimated that ~0.3% of the total protein membrane extract consisted of ThiT.

The oligomeric state of ThiT and other core transporters of the ECF-transporter family is unknown. The elution volume of ThiT from the size exclusion column, which had been calibrated with soluble globular protein standards, indicated that the molecular mass of the ThiT-DDM mixed micelle was ~100 kDa (data not shown). However, because the amount of detergent bound to the protein was not known, it was not possible to deduce the oligomeric state of ThiT in the ThiT-DDM micelle. Therefore, size exclusion chromatography coupled to static light scattering

(SEC-MALLS) was applied to ThiT (Figure 3A). A complication in these measurements was that empty DDM micelles cause peaks and troughs in the chromatogram of the light scattering measurement, which were not resolved from the ThiT-DDM mixed micelles on the size exclusion column, making calculation of the ThiT molecular weight in DDM unreliable. Peaks and troughs caused by empty micelles are commonly observed in SEC-MALLS measurements (*25*) and difficult to avoid. To overcome this problem, the detergent DDM was replaced with DM, which has a smaller micelle size (*25*, *27*, *28*). The results are depicted in Figure 3B. The elution peak of the ThiT-DM mixed micelle was now well resolved from the peak and trough caused by the empty detergent micelles. The molecular mass of ThiT in the DM micelle determined by the light scattering analysis was 22.7 kDa. As the molecular mass of ThiT calculated from the amino acid sequence was 21.2 kDa, it was concluded that ThiT was monomeric in the DM solubilized state.

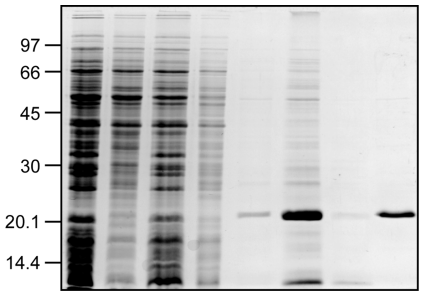

FIGURE 2: SDS−polyacrylamide gel stained with Coomassie blue of steps in a typical ThiT-nHis purification. From left to right: solubilized membrane vesicles (~40 μg of total protein loaded), flow-through after Ni-Sepharose binding, first wash fraction of the Ni-Sepharose column, second wash fraction, first elution fraction Ni-Sepharose column, second elution fraction, third elution fraction, and the peak fraction of the SEC (~0.7 μg of protein loaded).

Binding of Thiamin to Purified His_8-ThiT. ThiT contains five tryptophan residues, and the fluorescence emission spectrum of purified ThiT showed a maximum at 345 nm when excited at 280 nm (Figure 4). We intended to use changes in the intrinsic protein fluorescence to assay substrate binding to ThiT. However, the fluorescence signal did not change upon thiamin addition, regardless of the protein or substrate concentration (not shown). We hypothesized that a large proportion of the binding sites in the purified protein was already occupied with substrate at the beginning of the experiment. The source of the bound substrate could be thiamin from the complex growth medium (M17 broth) that had copurified with ThiT. Similar copurification of high-affinity ligands has been observed before in many different proteins (*29*−*31*). We used MALDI-TOF mass

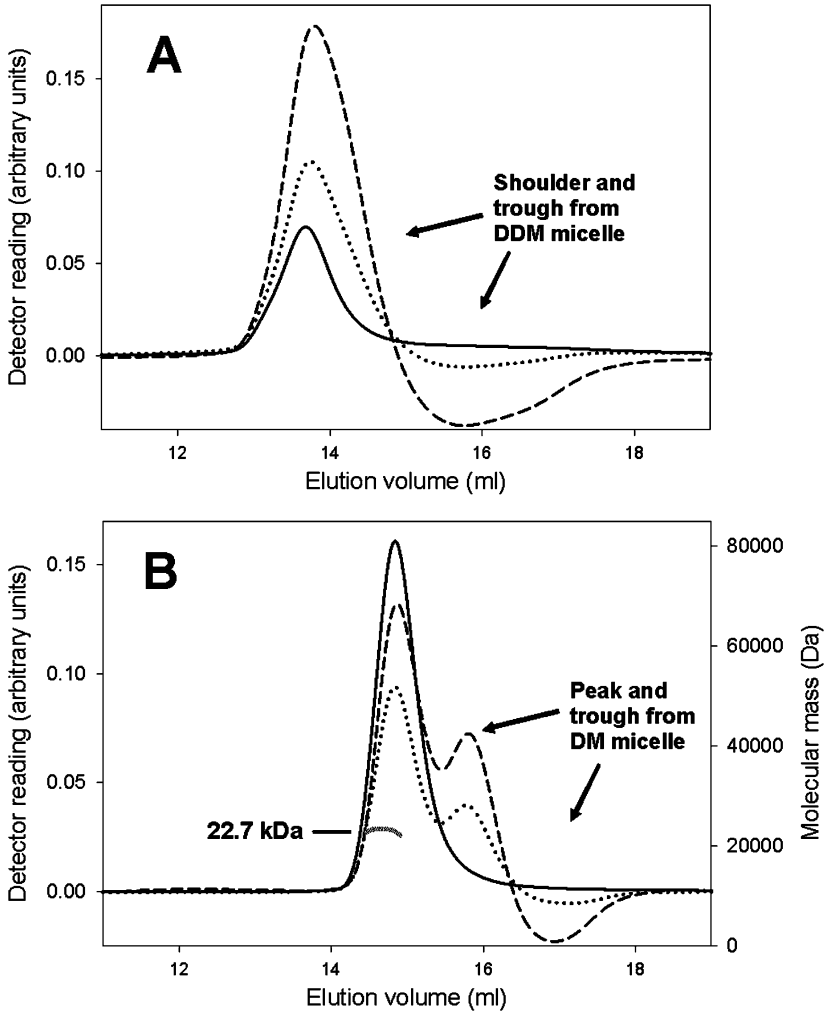

FIGURE 3: Determination of the ThiT oligomeric state. Chromatograms from size exclusion chromatography are shown for (A) ThiT purified in DDM and (B) ThiT purified in DM. Key: dashed lines, signal from the refractive index detector; dotted lines, signal from the static light scattering detector at 90°; solid black line, signal from absorption at 280 nm; solid gray line, calculated protein molecular weight (scale on right-hand *y* axis). The calculated average molecular mass for ThiT in DM was 22.7 kDa.

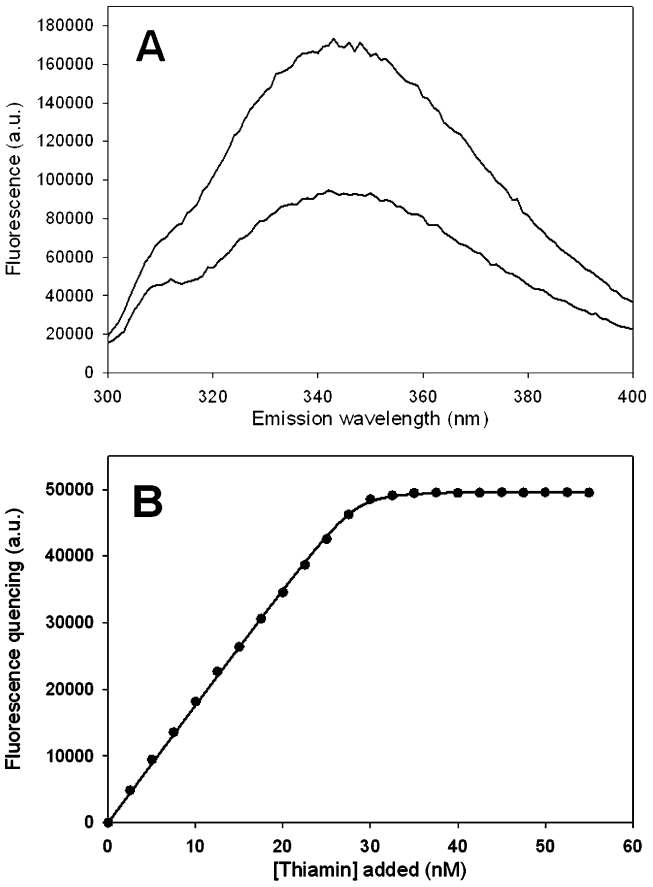

FIGURE 4: (A) Fluorescence emission spectra of substrate-free ThiT-nHis before and after titration with thiamin showing the large decrease in intrinsic tryptophan fluorescence. (B) Titration of substrate-free ThiT-nHis with thiamin. The dots represent the average fluorescene levels corrected for dilution measured at the indicated thiamin concentrations; the solid line is the best fit with eq 2.

spectrometry to find out whether indeed substrate was bound to purified ThiT. An intense signal at *m*/*z* 265.191 was detected in the mass spectrum of an extract of purified ThiT (Figure 5A) but not in control samples without ThiT. To assign this peak to thiamin (monoisotopic mass 265.112 Da), a reference spectrum was made from a thiamin−HCl solution (Figure 5B), in which a peak was observed at *m*/*z* 265.196, confirming that ThiT contained copurified thiamin.

The copurified thiamin prevented determination of the binding parameters, and therefore we aimed to obtain ligand-free ThiT by expressing the protein in cells that were cultivated in medium without thiamin. The ligand-free protein was purified, and the intrinsic tryptophan fluorescence was measured (Figure 4). The fluorescence was now strongly quenched in response to binding of thiamin, and the extent of quenching saturated at high thiamin concentrations (Figure 4). Typically, the fluorescence quenching at saturating thiamin concentrations was 47% (see Supporting Information Table 2). Quenching of tryptophan fluorescence thus reported binding and could be used to determine dissociation constants.

The dissociation constant K_D for thiamin binding to ligand-depleted ThiT was 122 ± 13 pM (Figure 4B, Table 1), and a binding stoichiometry of 0.84 ± 0.11 mol of thiamin/mol of ThiT was calculated. The observed stoichiometry was still lower than unity, which can be explained by errors in the protein concentration determination, a fraction of the purified ThiT being unable to bind substrate, or the presence of a small amount of residual

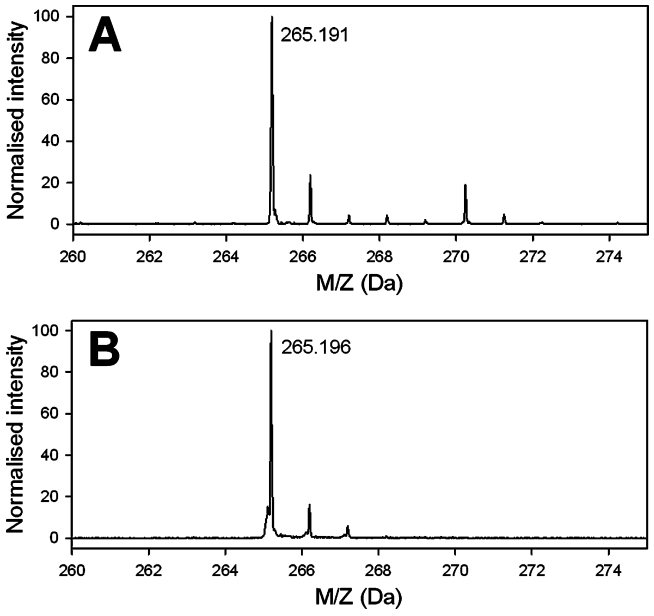

FIGURE 5: MALDI-TOF mass spectra of (A) ligand extracted from purified ThiT-His and (B) a thiamin−HCl solution. The monoisotopic mass of thiamin is 265.112 Da.

copurified substrate. Nonetheless, our data strongly suggest that there is one binding site per ThiT monomer. The fluorescence quenching assay was also used to determine K_D values for TMP and TPP binding (1.01 nM and 1.60 nM, respectively (Table 1)). The thiamin analogue pyrithiamin bound with a K_D value of 180 ± 70 pM (Table 1), whereas oxythiamin binding could not be observed. The presence of Mg^{2+} ions did not affect the affinity for TPP.

Binding of Thiamin to ThiT Mutants. A membrane topology model of ThiT is depicted in Figure 6. Six transmembrane domains are predicted with the N- and C termini located in the cytosol. Conserved residues are indicated in black, and a multiple sequence alignment of *L. lactis* ThiT and homologues is depicted in Supporting Information Figure 1. Genes encoding ThiT are found almost exclusively in Firmicutes. Because tryptophan fluorescence was strongly quenched upon thiamin binding (up to 47%; see Supporting Information Table 2), one or more tryptophan residues may be directly involved in binding. ThiT contains five tryptophans (indicated in gray in Figure 6), and to investigate their role in binding, they were replaced by alanines. Each mutant was overexpressed and could be purified in similar amounts as wild-type ThiT. The dissociation constants for thiamin, TMP, TPP, and pyrithiamin were determined using the fluorescence quenching assay (Table 1).

All tryptophan to alanine mutants were still capable of binding thiamin, but the W133A mutant had a dramatically reduced thiamin binding affinity (an increase in K_D of 3 orders of magnitude to 145 ± 20 nM) (Figure 7, Table 1). Tryptophan-133 is not strictly conserved in the ThiT family, but a tryptophan, phenylalanine, or tyrosine residue is always present at this position. To investigate whether the aromatic nature of tryptophan-133 was important for binding, the residue was replaced by phenylalanine. This substitution restored thiamin binding to wild-type values.

Quenching of the intrinsic protein fluorescence (26.9 ± 12.3%) was still observed in mutant W133A, indicating that other tryptophan residues also contributed to the substrate-dependent quenching. Since tryptophan-133 is located in a tryptophan-rich

Table 1: K_D Values (in nM) for Binding of Various Substrates to Wild-Type ThiT and Mutants[a]

	WT	W34A	W63A	W133A
thiamin	0.122 ± 0.013	0.35 ± 0.045	0.44 ± 0.095	145 ± 20
TMP	1.01 ± 0.14	3.79 ± 1.53	3.47 ± 0.01	2855
TDP	1.60 ± 0.00	4.24 ± 0.34	9.48 ± 3.62	5201 ± 406
pyrithiamin	0.180 ± 0.070	1.32 ± 1.25	0.23 ± 0.10	135 ± 12
	W138A	W141A	G129 V	G129A
thiamin	0.41 ± 0.015	0.09 ± 0.04	no binding	33.9 ± 3.95
TMP	3.57 ± 0.17	1.68 ± 0.75	no binding	810 ± 57
TDP	4.60 ± 1.70	1.93 ± 0.75	no binding	1792 ± 23
pyrithiamin	0.20 ± 0.05	0.10 ± 0.006	no binding	23.8 ± 2.35
	W133F	A137 V	Y146A	Y146F
thiamin	0.32 ± 0.13	0.27 ± 0.005	9.15 ± 0.55	0.44 ± 0.06
TMP	6.54 ± 0.24	3.55 ± 0.05	148 ± 5	4.8 ± 1.40
TDP	4.94 ± 0.07	5.23 ± 0.88	270 ± 30	7.8 ± 1.10
pyrithiamin	0.35 ± 0.11	0.16 ± 0.07	2.85 ± 0.35	0.24 ± 0.12
	S147A	N151A	N151S	W133A/Y146A
thiamin	0.23 ± 0.04	2.10 ± 0.21	2.1 ± 0.10	no binding
TMP	3.35 ± 0.95	38.7 ± 3.85	43.6 ± 5.00	no binding
TDP	5.80 ± 0.30	71.7	82.8 ± 6.10	no binding
pyrithiamin	0.15 ± 0.00	1.46 ± 0.02	1.55 ± 0.35	no binding

[a]The errors indicate standard deviations.

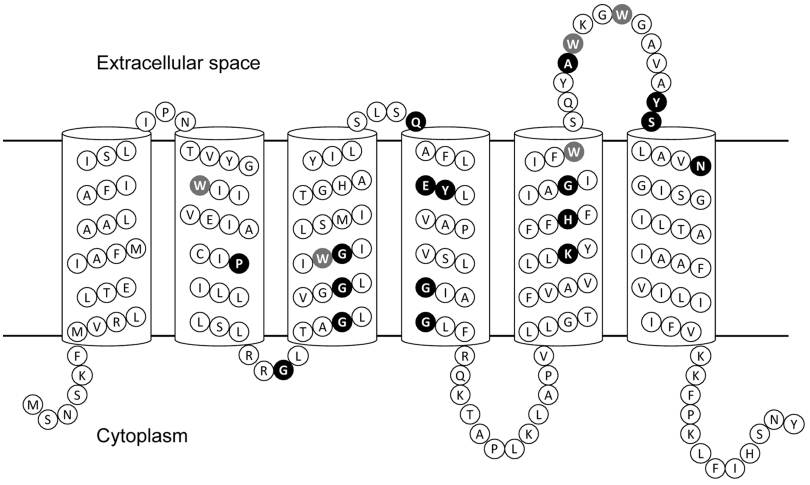

FIGURE 6: Graphical representation of the ThiT membrane topology model. The solid lines indicate the position of the lipid membrane. Tryptophans are depicted in gray and conserved amino acids in black.

region of the protein, we expected that the fluorescence of nearby tryptophan-138 and tryptophan-141 could also be affected by substrate binding. Surprisingly, all five tryptophans appear to contribute to the quenching upon thiamin binding (Table 1).

Three tryptophan residues, including tryptophan-133, are located in the region between transmembrane segments 5 and 6, which also contains several well-conserved residues (Figure 6). We thus hypothesized that this region may be important for thiamin binding. Five conserved amino acids (glycine-129, alanine-137, tyrosine-146, serine-147, and asparginine-151) were selected for mutagenesis (Table 1). All residues were changed to alanines with the exception of glycine-129 and alanine-137, which were replaced by a valine. The K_D values for the mutants A137V and S147A were very similar to that of the wild-type protein. In contrast, mutants Y146A and N151A had decreased binding affinities with K_D values for thiamin and the thiamin analogues of at least 1 order of magnitude higher than the wild-type protein (Figure 7 and Table 1). For G129V neither binding of thiamin nor of the analogues could be measured.

The reduced binding affinity of N151A could not be restored by introducing a different amino acid with a side chain that is available for hydrogen bond formation: Mutant N151S bound thiamin with a K_D value identical to that of N151A. In contrast, the reduced binding affinity of the mutant Y146A was restored to the wild-type value in the mutant Y146F (K_D = 440 ± 60 pM). The double mutant W133A/Y146A completely lost its ability to bind thiamin.

The strong conservation of glycine-129 and the total lack of thiamin binding to ThiT G129V demonstrated its importance. The incapability of G129V to bind thiamin might be explained by steric hindrance from the much larger valine side chain. To test this hypothesis, glycine-129 was replaced by the smaller alanine.

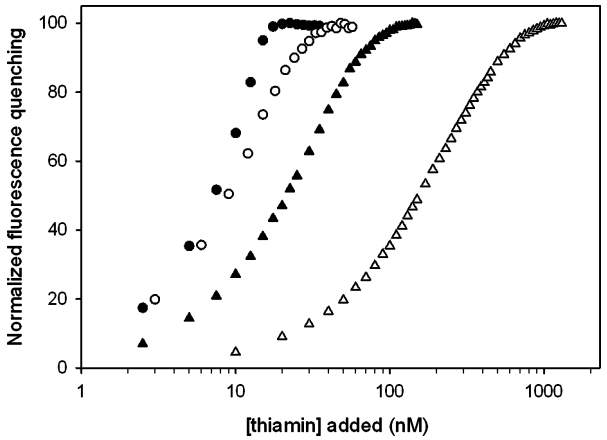

FIGURE 7: Semilogarithmic plot of thiamin binding curves for WT ThiT (filled circles), ThiT N151A (open circles), ThiT Y146A (filled triangles), and ThiT W133A (open triangles).

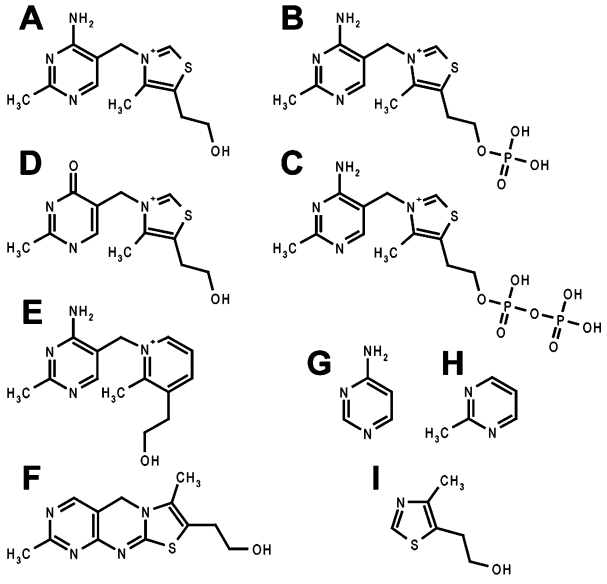

FIGURE 8: Chemical structures of the compounds used in this work: (A) thiamin, (B) TMP, (C) TPP, (D) oxythiamin, (E) pyrithiamin, (F) thiochrome, (G) 4-aminopyrimidine, (H) 2-methylpyrimidine, and (I) 4-methyl-5-thiazoleethanol.

In contrast to G129V, substrate binding to G129A could be measured, but the observed K_D values for binding thiamin and analogues were more than 100-fold higher than the values of wild-type ThiT.

Binding of Thiamin Analogues to ThiT. The thiamin molecule is built of two aromatic moieties: a thiazole and pyrimidine ring. Because both aromatic rings can contribute to binding via $\pi-\pi$ stacking interactions, individual thiazole and pyrimidine derivatives may also bind to ThiT. To test this hypothesis, binding of 4-aminopyrimidine, 2-methylpyrimidine, and 4-methyl-5-thiazoleethanol (Figure 8) to ThiT was assayed, but binding was not observed.

DISCUSSION

Thiamin binding to ThiT resulted in a strong decrease in the intensity of intrinsic tryptophan fluorescence, which must be associated with a change in environment of one or more tryptophan side chains (*32*). Binding of substrate could induce such changes either indirectly (as a result of global conformational changes upon substrate binding) or directly, when the indole side chain interacts with the thiamin molecule. In the latter case, substrate binding is expected to be severely affected by mutations that replace the tryptophan. Mutant W133A showed a decrease in binding affinity of 3 orders of magnitude, whereas mutation of none of the other tryptophans in ThiT affected binding significantly, indicating that W133 may be directly involved in thiamin binding. Tryptophan-133 is not strictly conserved between ThiT homologues, but an aromatic amino acid can be found at this position in all sequences. Because mutation of tryptophan-133 into phenylalanine did not result in a dramatic decrease in the binding affinity, we conclude that the aromatic nature of the side chain at position 133 is likely to be important for binding, with tryptophan-133 possibly contributing via $\pi-\pi$ stacking with either the pyrimidine or thiazole moiety of thiamin.

Tryptophan-133 is predicted to be located on the extracellular side of transmembrane helix 5, a region where several well-conserved residues are clustered. Site-directed mutagenesis and subsequent binding experiments of the conserved amino acids in this region identified glycine-129, tyrosine-146, and asparagine-151 as important for thiamin binding. Tyrosine-146 could, just as tryptophan-133, be replaced by phenylalanine without a significant change in binding affinity, and this residue may make a second aromatic contribution to thiamin binding, possibly via $\pi-\pi$ stacking with one of the thiamin aromatic rings. Since the thiamin molecule (Figure 8) includes two aromatic rings, we speculate that the rings interact with either tryptophan-133 or tyrosine-146. The incapability of ThiT to bind the nonaromatic oxythiamin further emphasizes the importance of tryptophan-133 and tyrosine-146 in the ThiT binding site. In addition, the double mutant W133A/Y146A completely lost its ability to bind thiamin. In contrast to all other mutants the Y146A mutant is more adversely affected in thiamin binding than in pyrithiamin binding. It is possible that tyrosine-146 interacts with the thiazole ring of thiamin, which in pyrithiamin is replaced by a pyridine. A model of the most important interactions in the ThiT binding site is depicted in Figure 9.

There are many structures available of soluble TPP-dependent enzymes and thiamin binding proteins. Examples are pyruvate dehydrogenase (*33*), benzoylformate decarboxylase (*34*), and the ABC transporter substrate binding proteins TbpA from *Escherichia coli* (*7*) and CypL from *Mycoplasma hyorhinis* (*35*).

The TPP-dependent enzymes often show a remarkably similar three-dimensional structure, despite low sequence similarity (*36*). The TPP aminopyrimidine ring is often bound in a hydrophobic pocket and can be involved in π-stacking with aromatic amino acids, which we also hypothesize for ThiT. In all TPP-dependent enzymes, TPP adopts the so-called "V-conformation" (*37*). In this conformation the N1 nitrogen of the aminopyrimidine ring is held in the proximity of a conserved glutamate and the 4-amino group is oriented toward the C2 carbon of the thiazole ring, thus stabilizing the TPP transition state during catalysis. The main interaction for TPP binding is provided by the diphosphate group that is coordinated by a divalent cation (often Mg^{2+}), ensuring high specificity for TPP over TMP or thiamin. The Mg^{2+} ion is coordinated by an aspartic acid/glutamic acid that is part of a conserved thiamin binding motif (*38*). In contrast, TPP binding to ThiT does not require Mg^{2+} and TMP, and thiamin can bind more tightly than TPP; in addition, ThiT does not appear to have the thiamin binding motif. Therefore, we expect the ThiT binding site to be different from that of TPP-dependent enzymes.

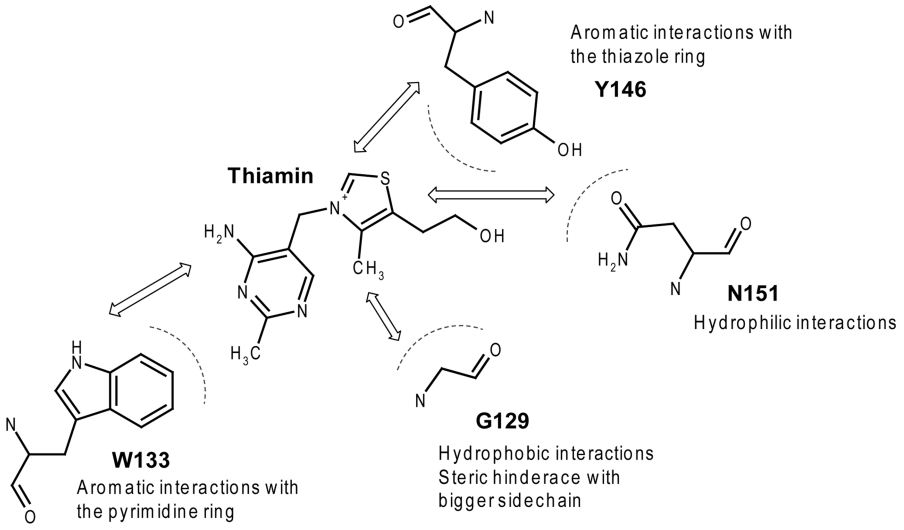

FIGURE 9: Model of the ThiT binding site. Interactions between thiamin and the conserved residues which when mutated strongly affected the affinity are depicted.

The thiamin binding protein TbpA was cocrystallized with TMP in its binding site. The thiazole ring of TMP is positioned between two tyrosines, neither of which is involved in π-stacking. A third aromatic residue, tryptophan-197, was located close to the pyrimidine ring. Like ThiT, TbpA does not require divalent cations for the binding of TMP or TPP. Instead, the phosphate group of TMP is coordinated by hydrogen bonds to tryptophan-197, serine-161, glycine-60, and aspartate-59 rather than through cation coordination. Thiamin binds with approximately equal affinity to TbpA as TMP does, even though there is no phosphate moiety available for interactions. Modeling of thiamin in the binding site suggested that it adopts an alternative conformation which enables π-stacking of tryptophan-197 and tyrosine-27 to the pyridine and thiazole moiety, in this way possibly compensating the loss of favorable phosphate interactions. This is a similar pattern of π-stacking as proposed for ThiT. It is estimated that each π-stacking can contribute -14.7 kJ/mol of free energy to binding (*39*), which is significant compared to the -56.9 kJ/mol of free energy change that is associated with an equilibrium dissociation constant of 100 pM, as observed for ThiT. π-Stacking interaction may therefore be an important contribution to high-affinity thiamin binding.

The overall structure of CypL is comparable to that of TbpA, despite low sequence similarity. There are nonetheless big differences between both binding sites. In the CypL crystal structure, a TPP molecule is clearly resolved. The phosphates of TPP are coordinated with two Ca^{2+} ions. The amino acids that form the Ca^{2+} binding sites in CypL are absent from the TbpA sequence. TPP is not stacked with aromatic residues, but tryptophan-314 and tyrosine-343 are oriented such that π-stacking with the pyrimidine and thiazole rings is possible if TPP is bound in a different conformation. This is very similar to the proposed thiamin binding to TbpA and might also be an important feature of the ThiT binding site. Despite the agreement between the thiamin binding sites of soluble thiamin binding proteins and the experimental results presented in this work, it is important to note that there is no structural information available of any thiamin binding *membrane* protein. A global structural resemblance between ThiT and the soluble proteins discussed here is very unlikely.

The dissociation constant of ThiT for thiamin is at 100 pM remarkably low. Such high affinity is unusual for membrane transport proteins. A similar affinity has been reported for the riboflavin transporter RibU from *L. lactis* (*29*). Although RibU and ThiT do not have sequence similarity, they are similar in hydrophobicity, molecular weight, and number of predicted transmembrane segments. Moreover, RibU and ThiT are both core transporters of the ECF class of membrane transporter proteins (*10*). Other core transporters for folate, thiamin, and biotin from *L. casei* (*9*, *40*−*42*) also bind their respective substrates with high (nanomolar) affinity. We therefore hypothesize that high-affinity substrate binding is a general characteristic for ECF transporter family proteins and related to their mechanism of transport. Such a transport system would be most valuable when only very little amounts of vitamin are available, and it is exactly under these conditions that the proteins are maximally expressed as a result of their riboswitch regulation. The core transporters are then able to scavenge the low abundant vitamins and transport them to the cytosol.

The ECF core transporters bind their substrates with such high affinity that substrate release on the cytoplasmic side of the membrane might become problematic and would most likely require input of additional energy. This energy can be provided by the tripartite ECF complex by means of ATP binding and hydrolysis. We therefore propose a model for the ECF transport mechanism in which the core transporters (substrate binding proteins, e.g., ThiT) interact with the tripartite ECF complex if high rates of substrate translocation are required, whereas the core transporters alone can directly mediate low flux substrate translocation in the absence of the ECF proteins. This model does not explain the observations made for the kinetics of biotin transport through the BioMNY ECF transporter from *Rhodobacter capsulatus* (*13*). However, the genes for the BioMNY system are located in a single operon, and the three encoded proteins are believed to form a dedicated complex for the transport of biotin. In wild-type strains BioY may always be present in a complex with BioM and BioN, and a separate function for the solitary BioY protein is therefore not expected. The model presented above might thus not apply to BioMNY but only to ECF transporters in which the tripartite ECF module is shared by many different core transporters.

Among the strictly conserved residues in the ThiT homologues, there is a GxxxG motif repeat in the third predicted

transmembrane segment. Not only in ThiT but also in other ECF family core transporters are the motifs observed. These motifs are well-known to promote association in membrane proteins by stabilizing helix−helix interactions in the membrane (*43*). A possible explanation would be that the GxxxG motif provides an opportunity for interacting with the ECF transmembrane subunit. Alternatively, the GxxxG motif could serve as a platform for self-association of ThiT. Size-exclusion chromatography coupled to static light scattering and refractive index measurement, however, unambiguously showed that ThiT is a monomer in detergent solution. Nevertheless, it cannot be excluded that in the membrane ThiT is an oligomer which it falls apart during detergent solubilization, because it adopts a non-native conformation under these conditions. However, such dissociation seems unlikely because monomeric ThiT in detergent solution is capable of high-affinity substrate binding.

In Gram-negative bacteria, substrate scavenging by ABC transporters is accomplished by periplasmic substrate binding proteins (*44*, *45*) which deliver their substrates to ABC transporters residing in the inner membrane. In Gram-positive bacteria it is often observed that the substrate binding proteins are genetically fused to their corresponding ABC transporter or covalently attached to membrane lipids (*46*, *47*), a phenomenon that is linked to the lack of a confined periplasm. For similar reasons, the ECF transporter class might be particularly suited for Gram-positives, which is in agreement with their phylogenetic distribution, as the ECF transporters are particularly abundant in Gram-positive organisms (*10*).

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SUPPORTING INFORMATION AVAILABLE

Details on the construction of the expression vectors, the sequences of the oligonucleotides used, a multiple sequence alignment of ThiT and homologues, and a table with details on the fluorescence quenching of the five tryptophan mutants by thiamin. This material is available free of charge via the Internet at http://pubs.acs.org.

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