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ATP binding drives substrate capture in an ECF transporter by a release and catch mechanism

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Abstract

ECF transporters are a family of active transporters for vitamins. They are composed of four subunits: a membrane-embedded substrate-binding subunit (EcfS), a transmembrane coupling subunit (EcfT), and two ATP-binding cassette ATPases (EcfA and EcfA'). We have investigated the mechanism of the ECF transporter for riboflavin from the pathogen *Listeria monocytogenes*, LmECF–RibU. Using structural and biochemical approaches we find that ATP binding to the EcfAA' ATPases drives a conformational change that dissociates the S subunit from the EcfAA'T ECF module. Upon release from the ECF module, the RibU S subunit then binds the riboflavin transport substrate. We also find that S subunits for distinct substrates compete for the ATP-bound state of the ECF module. Our results explain how ECF transporters capture the transport substrate and reproduce the *in vivo* observations on S subunit competition for which the family was named.

Energy coupling factor (ECF) transporters are a family of primary active membrane transporters for the uptake of essential micronutrients, such as vitamins and trace metals¹. Found exclusively in bacteria and archaea, ECF transporters were initially identified in bacteriological experiments on *Lactobacillus casei*. These studies found that the ATP-dependent uptake of folate was reduced in the presence of the structurally unrelated thiamine^{2–4}. Consequently, the authors proposed that distinct binding proteins for the respective vitamins, the S subunits, utilize a common means for coupling cellular energy to transport, the energy coupling factor. These observations in whole cells suggested a dynamic association of the ECF and the S subunit and that distinct S subunits compete for the same ECF module⁴. Similarly, the transport mechanism of ATP-binding cassette (ABC) importers involves dynamic association of the transmembrane subunits with a substrate binding protein⁵.

Roughly 30 years later, bioinformatics and biochemical studies^{1,6} determined that the active transporter is formed by a high affinity integral membrane substrate-binding subunit (EcfS)

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The atomic coordinates and structure factors for the crystal structure of the AMPPNP-bound EcfAA' heterodimer from *T. maritima* have been deposited in the Protein Data Bank as 4ZIR.

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in complex with an energy coupling factor composed of a transmembrane coupling subunit (EcfT) and two homologous ABC ATPases (EcfA and EcfA)⁶. As a result, ECF transporters can be considered a unique sub-family within the ABC transporter superfamily of ATP-dependent pumps^{7,8}. In group I ECF transporters, the four subunits form a dedicated complex for the uptake of a single substrate, as typified by the biotin transporter from *Rhodobacter capsulatus*⁶. In contrast, S subunits for distinct substrates share a common ECF module in the group II members¹. For example, in *L. lactis*, S subunits for eight different vitamins utilize the ECF transporter platform to import their substrates⁹. In both groups, ATP-driven conformational changes in the ECF module are presumably coupled to binding and release of the substrate from EcfS.

The first crystal structures of a complete ECF transporter¹⁰⁻¹² revealed the subunits associated in a 1A':1A:1T:1S quaternary architecture as anticipated from solution studies on ECF transporters from *L. lactis*⁹. The nucleotide-free EcfAA' ATPase subunits were observed in an open conformation. EcfT displayed a novel L-shaped fold in which a parallel bundle of five TM helices is roughly perpendicular to three cytoplasmic helices (CH1-3). The S subunit in each structure interacts exclusively with EcfT. Unexpectedly, the substrate-binding site is facing the cytoplasm, as if the entire EcfS subunit has been "toppled" from an extracellular-facing, substrate-capturing orientation. As a result, these crystal structures may have captured a post-translocation state in which nucleotides had dissociated and the transport substrate was released into the cytoplasm.

The ECF transporter structures suggested a novel mechanism for active translocation of a small molecule across the lipid bilayer^{13,14}. Instead of opening a transient pathway for substrate at the EcfS-T interface, ECF transporters appear to couple the binding and hydrolysis of ATP to rotation of EcfS through the membrane at the subunit interface. While these crystal structures were consistent with the available data on ECF transporter assembly^{9,15-17}, there is no biochemical evidence supporting this "toppling" mechanism. Furthermore, in order for EcfS to capture substrate, the subunit must reorient to expose the substrate-binding site to the extracellular space. Potentially, ATP binding could catalyze reorientation of EcfS to an extracellular-facing state, leading to substrate capture within an intact transporter complex. Alternatively, ATP could drive dissociation of the S subunit from the ECF module, followed by substrate binding by EcfS in isolation. Given these two disparate hypotheses, we set out to determine the role of ATP binding and the mechanism of substrate capture by an ECF transporter.

RESULTS

We expressed and purified a stable complex between the EcfAA'T ECF module and the riboflavin binding S subunit, RibU, from the human pathogen *Listeria monocytogenes*. Unlike solitary RibU^{18,19}, the LmECF-RibU complex did not co-purify with the riboflavin transport substrate as evidenced by the lack of an absorbance in the 400-500 nm range. In detergent solution, LmECF-RibU eluted from size exclusion chromatography (SEC) as a single sharp symmetrical peak (Supplementary Fig. 1a). Analysis of this sample by multi-angle laser light scattering²⁰ indicated a molecular mass of 117 ± 3.9 kDa for the protein component of the protein-detergent complex (Supplementary Fig. 1b). An LmECF-RibU

complex containing one copy of each subunit has a predicted molecular mass of ~116 kDa with individual subunit masses of 31.3, 32.0, 30.2, and 22.7 kDa for EcfA', A, T, and RibU, respectively. Therefore, these data are consistent with the 1A':1A:1T:1S subunit stoichiometry that was observed in the purified *L. lactis* ECF transporters⁹ and the crystal structures¹⁰⁻¹².

The S subunit is in a “toppled” conformation in LmECF–RibU

In the “toppled” conformation observed in the ECF transporter crystal structures¹⁰⁻¹² the extracellular loops that connect transmembrane (TM) helices TM3 and 4 (Loop 3-4) and TM5 and 6 (Loop 5-6) of EcfS are buried at the interface with EcfT. Loop1-2, which encloses the bound substrate in the S subunit crystal structures^{15,19,21}, has retracted to expose the substrate binding site to the cytoplasm. In order to determine whether the S subunit in LmECF–RibU also adopts a “toppled” orientation in the nucleotide-free state, we determined the solvent accessibility of the extracellular loops of RibU both in isolation and in the complete transporter complex (Supplementary Note 1). As wild-type (WT) LmRibU lacks cysteine residues, we introduced a single cysteine into two positions in each of the three extracellular loops. Subsequently, the solvent exposure of these sites was determined via reaction with the thiol reactive fluorophore, tetramethylrhodamine-5-maleimide (TMRM)^{22,23}. Based upon an LmRibU homology model (Figure 1a), all of these sites were expected to react with TMRM. Consistently, the purified LmRibU L29C and P34C mutants in Loop1-2 readily reacted with the probe in solution (Fig. 1b and Supplementary Fig. 2). Similarly, cysteine substitutions of Ser72 and Ser74 in Loop3-4, and Ile137 and Gly140 in Loop5-6 were also found to be solvent accessible in LmRibU (Fig. 1b). In contrast, the L25C mutant, which is located in TM1 and should be shielded by the detergent micelle in the purified protein, displayed reduced reactivity compared to the most accessible site in each extracellular loop. However, the origin of the detailed accessibility differences within these loops will need to be addressed by further investigation.

While bound to the ECF module in the complete transporter complex (Fig. 1c), the extracellular loops of LmRibU (Fig 1c.) displayed a dramatically different pattern of TMRM accessibility. The labeling of S72C and S74C in Loop3-4 and I137C and G140C in Loop5-6 is reduced by two orders of magnitude when in complex with ECF module (Fig. 1d, Supplementary Fig. 3). The L25C and P34C mutants in Loop1-2 reacted with TMRM, but the labeling of P34C is reduced by ~80% in the full transporter compared to riboflavin-bound LmRibU in isolation. This observation is consistent with the increased mobility of Loop1-2 in the substrate-free conformation^{10-12,24}. Although these data do not define the relative orientation of RibU in the transporter complex, the pattern of labeling is completely consistent with the ECF transporter crystal structures in which Loops3-4 and 5-6 of EcfS are buried at the interface with EcfT, while Loop1-2 is solvent exposed in the cytoplasm (Fig. 1c). In addition, a mutational study that probed the EcfT–EcfS interface was consistent with the available ECF transporter structures¹². Taken together, our findings suggest that in the absence of nucleotide and transport substrate, LmECF–RibU is predominantly in the ATPase-open, toppled conformation.

Crystal structure of EcfAA' bound to AMPPNP

We previously determined the structure of the EcfAA' heterodimer from *Thermotoga maritima* (TmEcfAA') bound to the hydrolysis product, ADP (Fig. 2a). The structure revealed the ATPase domains in an open conformation, in which the heterodimer associates via the carboxy-terminal subdomains²⁵. To gain insight into the role of ATP binding in ECF transporters, we determined the structure of TmEcfAA' in the presence of the non-hydrolyzable ATP analog AMPPNP to a resolution of 3.0 Å (Table 1). In this structure, AMPPNP binding caused a transition to a closed conformation in which two AMPPNP molecules are buried between the P-loop and LSGGQ motifs in each subunit (Fig. 2b). Relative to the ADP-bound, open conformation, the two ATPase domains undergo a ~5 Å translation towards one another to form the closed state. This AMPPNP-driven domain closure mirrors that observed in other ABC-type ATPases, such as the MalK homodimer of the *E. coli* maltose transporter²⁶⁻²⁸.

In order to perform productive mechanical work, the ATP-driven transition in EcfAA' must be coupled to conformational changes in EcfT. ECF-type ATPases share a structural motif called the Q-helix located between the core and helical subdomains²⁵. This motif consists of a short helix containing an invariant glutamine, with the consensus sequence of xPD/ExQφ (φ is a hydrophobic residue). In the ECF transporter crystal structures, the Q-helices of EcfA and EcfA' directly interact with the EcfT coupling helices CH2 and CH3, respectively¹⁰⁻¹². In the open ATPase structures, the Q-helices are ~12 Å apart and do not contact the nucleotide (Fig. 2c, Supplementary Table 1, Supplementary Note 2). In contrast, this distance is reduced to 7 Å in the closed structure of TmEcfAA', and the Q-helices directly interact at the subunit interface (Fig. 2d). Glu88 and Asp89 of EcfA and Asn80 and Ser82 of EcfA' form a local cluster of hydrogen bonds at the center of the dimer. Consequently, the Q-helices are responsible for attachment to the coupling helices as well as stabilization of the ATP-bound, closed state.

Given the extensive interface between the EcfT coupling helices and the ATPase subunits^{10,11}, the ATP-driven domain closure of EcfAA' may produce a substantial movement of the attached CH2 and CH3. To investigate this hypothesis, the nucleotide-free ECF folate transporter structure¹⁰ (Fig. 2e) was placed in the closed conformation captured in AMPPNP-bound TmEcfAA'. This modeling exercise suggests that upon ATP binding to form the closed state, CH2 and CH3 slide along one another while maintaining contact with the Q-helices. Therefore, instead of closing like a pair of scissors¹³, ATP binding pushes CH2 and CH3 past one another. As EcfS sits directly on top the coupling helices in the crystal structures, this conformational transition may disrupt the interaction with EcfT (Fig. 2f). Thus, the ATP-bound state of the ECF module may have reduced affinity for the S subunit, whereby EcfS could either reorient towards the extracellular space or dissociate from the ECF module altogether. Upon ATP hydrolysis, which is required for transport²⁵, the ATPase domains would return to the open state observed in the crystal structures (Fig. 2a).

Trapping the ATP-bound closed conformation of LmECF–RibU

The TmEcfAA' crystal structures suggest that ATP binding may be coupled to dissociation of the S subunit from the ECF module. In order to test this hypothesis in the complete transporter, it is essential to separate the role of ATP binding from its hydrolysis. Therefore, we conducted a series of experiments to trap an ECF transporter in the ATP bound, pre-hydrolysis conformation. In detergent solution, purified LmECF–RibU hydrolyzes ATP with a $V_{\max}$ of 1150 ± 28 nmol/min/mg protein with a K_m of 165 ± 16 μ M (Fig. 3a). In proteoliposomes, LmECF–RibU hydrolyzed ATP with a $V_{\max}$ of 488 ± 67 nmol/min/mg, approximately 50% of the activity observed in detergent (Fig. 3b). As the transporter probably inserts into the membrane in a random orientation upon reconstitution, only one-half of the transporter molecules will be available to hydrolyze the externally added ATP in this assay. These data along with the monodispersity of our transporter preparation (Supplementary Fig. 1) indicate that the purification of LmECF–RibU in detergent does not impact the activity of the transporter.

As AMPPNP and vanadate were poor inhibitors of the LmECF–RibU ATPase activity (Fig. 3b, Supplementary Fig. 4, Supplementary Note 3), we used a mutational strategy to arrest the transporter in the ATP-bound, pre-hydrolysis conformation. Previous studies established that an invariant histidine in the “H- or ‘switch-loop” is essential for ATP hydrolysis in ABC-type ATPases²⁹⁻³¹. In ATP-bound crystal structures, this histidine directly contacts the γ -phosphate of ATP (Fig. 3c). Mutation of this residue to alanine in the α -hemolysin ABC transporter, HlyB, abolished the ATPase activity without a reduction of the ATP binding affinity²⁹. As a result, binding of MgATP to this H-loop mutant trapped the ATPase domains in a closed, dimeric, pre-hydrolytic state^{32,33}. Thus, to construct a hydrolysis deficient mutant of an ECF transporter, we mutated both His199 of LmEcfA' and His204 of LmEcfA to alanine to generate a dual H-loop mutant. These mutations reduced the ATPase activity of purified LmECF–RibU to the background levels observed in the absence of the Mg^{2+} cofactor (Fig. 3b). Importantly, the H-loop mutations did not have an adverse effect on the stability of the transporter complex (Supplementary Fig. 5). Therefore, we had successfully isolated an ATPase deficient ECF transporter that was stable in detergent solution.

ATP binding drives RibU dissociation from the ECF module

Our structural data suggests that ATP binding to the nucleotide-free transporter disrupts the tight interaction between the ECF module and the inward-facing S subunit. To investigate this hypothesis, we tested the effect of different nucleotides on the oligomeric state of both the WT and hydrolysis deficient LmECF–RibU transporters in solution. In the absence of nucleotide, the WT transporter eluted as a single symmetrical peak during analytical SEC (Fig. 4a). Pre-incubation of the sample with the hydrolysis product MgADP did not have a detectable effect on the stability of the transporter complex. This result is consistent with the structural data, whereby the nucleotide-free and ADP bound states of the transporter adopt a similar ATPase-open conformation^{10-12,25,34} with a 1:1:1:1 subunit stoichiometry⁹ (Supplementary Fig. 1). Similarly, MgATP, which is readily hydrolyzed by the WT protein (Fig. 3a), did not alter the quaternary structure of the transporter in this assay (Fig. 4a).

In the absence of nucleotide or upon incubation with MgADP, the ATP hydrolysis deficient H199A' H204A mutant behaved identically to the WT protein in SEC. In contrast, binding of MgATP resulted in a dramatic change in the SEC profile whereby the transporter peak dissociated into two peaks (Fig. 4b). Notably, the position of the second peak corresponds to the elution profile of solitary LmRibU, and is too large to be the EcfAA' heterodimer (Fig. 4a). As a result, these data indicate that the ATP-driven domain closure observed in the TmEcfAA' crystal structures is coupled to disruption of the EcfS-T interface, leading to dissociation into the EcfAA'T sub-complex and free RibU.

To verify that ATP binding is responsible for dissociation of the S subunit from the ECF module, we varied the MgATP concentration during the incubation step with the H199A' H204A mutant prior to analytical SEC. As expected, the height of the RibU peak increased with MgATP concentration, while the height of the LmECF-RibU peak decreased (Fig. 4c), which is consistent with MgATP binding promoting dissociation of the S subunit. Analysis of these data indicates a K_d of $101 \pm 19 \mu\text{M}$ for MgATP binding, which is in agreement with the K_m of ATP hydrolysis by LmECF-RibU.

Free RibU captures the riboflavin transport substrate

Our analysis of the quaternary structure of LmECF-RibU indicates that ATP binding disrupts the tight complex between the ECF module and the RibU S subunit. This observation suggests that the S subunit may capture transport substrate in isolation and dynamically associate with the ECF module during the membrane transport cycle. To test this hypothesis, we monitored riboflavin (vitamin B2) binding to LmECF-RibU by utilizing the B2 intrinsic fluorescence and fluorescence size exclusion chromatography (FSEC)³⁵. Pre-incubation of the H199A' H204A mutant of LmECF-RibU with riboflavin had no effect on the oligomeric state of the transporter (Fig. 4e) and no evidence of riboflavin binding was detected in the FSEC trace (Fig. 4f). In contrast, pre-incubation with both riboflavin and MgATP, which drives RibU dissociation (Fig. 4e), revealed a riboflavin fluorescence signal associated with the free RibU peak (Fig. 4f). Consequently, upon ATP-driven ejection from the ECF module, the free S subunit binds the transport substrate. Indeed, multiple studies have indicated that S subunits display higher substrate binding affinity in isolation from the ECF module^{18,21,36,37}. We also found that AMPPNP binding to WT LmECF-RibU releases RibU from the ECF module (Supplementary Fig. 4b). However, this effect requires higher concentrations of AMPPNP, as expected from the relatively low K_i (Supplementary Fig. 4a). Importantly, we only detected riboflavin binding to the dissociated RibU S subunit and not to the full transporter (Supplementary Fig. 4b-c). Therefore, these data are inconsistent with a mechanism of ATP-driven cycles of EcfS “toppling” within the transporter complex. Instead, our results suggest a “release and catch” mechanism, whereby ATP binding dissociates the complex between the ECF module and RibU, allowing the released S subunit to capture substrate from the extracellular environment.

S subunit competition for the ECF module is MgATP-dependent

The modularity of group II ECF transporters raises the possibility that a specific EcfAA'T exchanges different S subunits during the transport cycle. We tested whether LmECF-RibU could exchange one S subunit molecule for another using the experimental approach

described in Supplementary Fig. 6. Equimolar mixtures of WT LmECF–RibU and TMRM-labeled RibU (hereafter referred to as RibU*) were incubated with no nucleotide, MgADP, or MgATP, and analyzed by FSEC (Fig. 5a-b). As expected, RibU* displayed a single FSEC peak while LmECF–RibU was not fluorescent at these wavelengths (Fig. 5b). However, a fluorescent peak corresponding to the full transporter is present after incubation with MgATP, suggesting that during the binding and hydrolysis of ATP, RibU* has replaced the endogenous RibU subunit in the ECF module. As the elution position of the transporter does not change (Fig. 5a), this precludes the spurious incorporation of additional copies of RibU* to LmECF–RibU. Furthermore, we exclude the possibility that the observed FSEC peak is due to transfer of the label between RibU* and LmECF–RibU (Supplementary Fig. 7). Examination of the FSEC peak by SDS-PAGE and fluorescence scanning (Fig. 5c) indicates that the endogenous RibU has been exchanged for RibU*.

Our “release and catch” model predicts that S subunit exchange by the ECF module depends on the amount of available EcfS. We found that increasing the RibU* concentration in the ATP-dependent exchange reaction with LmECF–RibU leads to saturation of the ECF module, with a $B_{\max}$ of 41.7 ± 9.6 pmol and an apparent K_d for RibU* of 6.4 ± 2.3 μ M (Fig. 5d). The saturation of the binding curve agrees with the amount of injected LmECF–RibU, as 100% recovery from the column would correspond to 45 pmol of transporter (Methods). Notably, the affinity of the ECF module for RibU is ~1000-fold lower than the K_d of isolated RibU for the riboflavin transport substrate¹⁸. In this way, a “release and catch” transport mechanism exploits the high substrate binding affinity of EcfS while the lower intersubunit affinity would facilitate S subunit competition. However, the EcfAA'T–EcfS interaction may be of higher affinity *in vivo*, where the membrane environment confines the diffusion between the subunits to two dimensions.

A transport model involving EcfS exchange predicts that S subunits for different vitamins will compete for the ECF module, and such competition has been suggested by vitamin uptake experiments⁴ and co-purification experiments from native hosts⁹. To investigate S subunit competition *in vitro*, we purified a polyhistidine-tagged variant of the thiamine binding S subunit from *L. monocytogenes*, ThiT, and determined the amount of RibU* exchange in the presence of increasing concentrations of ThiT. The ATP-dependent S-subunit exchange by LmECF–RibU is inhibited in the presence of ThiT in a concentration dependent manner (Fig. 5e), suggesting that ThiT has bound the ECF module in place of RibU*. To test this hypothesis, we collected the fluorescent transporter peak fraction from the “red” experiment in Fig. 5e and visualized the contents by Western blotting for the polyhistidine-tag on ThiT (Fig. 5f). These data show that ThiT has been incorporated into the transporter, accounting for the reduced exchange of RibU*. These findings explain the *in vivo* observations on S subunit competition for the ECF module for which the Energy Coupling Factor transporters were named.

S subunits exchange with the ATP-bound ECF module

Our data indicate that LmECF exchanges S subunits by an ATP-dependent mechanism. As ATP binding is coupled to dissociation of the substrate-free, “toppled” RibU S subunit (Fig. 4b), we tested whether this ATP-bound conformation could bind to a distinct, substrate-

bound S subunit. Equimolar mixtures of H199A H204A LmECF–RibU and RibU* were incubated with no nucleotide, MgADP, or MgATP, and analyzed by FSEC. Only MgATP binding, which traps this mutant in the ATP-bound, closed state, leads to incorporation of RibU* in this assay (Fig 6a). Increasing the RibU* concentration leads to saturation of the ATP bound ECF module, with a $B_{\max}$ of ~25 pmol and an apparent K_d for RibU* of ~8 μ M (Fig. 6b). Consequently, these data demonstrate that the ATP-bound ECF module can bind to a distinct substrate-bound S subunit.

DISCUSSION

ATP binding drives substrate capture by S subunit dissociation

Although EcfS subunits exhibit high binding affinity for the transport substrate in isolation^{18,19,21,36,37}, several lines of evidence indicate that this affinity is reduced in complete ECF transporters. First, while isolated S subunits co-purify with their respective transport substrate^{15,18,19,21} complete ECF transporters do not^{9-12,25}. Second, attempts to co-crystallize the *L. brevis* ECF transporters with substrate by multiple labs were unsuccessful¹⁰⁻¹². Third, we observed no evidence of high affinity substrate binding by the LmECF–RibU transporter (Fig. 4f). However, *in vivo* ECF-mediated folate uptake displayed a K_m of ~40 nM, which is consistent with the high binding affinity of a solitary S subunit³. Our findings resolve these contrasting mechanistic details by demonstrating that ATP binding drives dissociation of the S subunit from the ECF module. Once free from the ECF module, RibU binds the riboflavin transport substrate (Fig. 4e-f). These results define the role of ATP binding and explain how ECF transporters capture the transport substrate from the extracellular milieu.

S subunits compete for the ECF module

We have found that ATP binding induces a conformation of the ECF module that allows exchange of S subunits. While this state has reduced affinity for the “toppled” S-subunit relative to the nucleotide-free conformation (Fig. 4b), it can readily bind to a distinct substrate-bound EcfS protein (Fig. 6). Indeed, the competition of S subunits for the ECF module is substrate-dependent *in vivo*, which suggests that substrate-bound and substrate-free S components have different affinities for the ECF module^{4,14}. The large conformational change of Loop1-2 upon substrate binding²⁴ could present a unique surface for interaction with EcfT compared to an empty EcfS. Similarly, the maltose binding protein undergoes an open-to-closed transition upon substrate binding, and this closed state stimulates ATP hydrolysis of the maltose ABC transporter^{38,39}. Therefore, this “release and catch” mechanism would maximize the efficiency of transport through the selective recognition of substrate-loaded EcfS by the ECF module. In addition, through the regulated expression of specific S subunits^{37,40-42}, cells could readily adapt to differences in substrate availability while utilizing a single transporter platform.

Transport mechanism for group II ECF transporters

The available structural, biochemical, and bacteriological data suggest a membrane transport mechanism for group II ECF transporters (Fig. 7). In the absence of nucleotides and transport substrate, the transporter adopts an ATPase-open conformation, in which the

substrate-binding site of EcfS is open to the cytoplasm. This state has been captured in crystal structures of the *L. brevis* ECF module in complex with three different S subunits¹⁰⁻¹², and is supported by our accessibility studies of LmECF–RibU (Fig. 1). ATP binding to EcfA and A' drives a transition to the closed state, as observed in the TmEcfAA' crystal structures²⁵ (Fig. 2). This conformational change slides the EcfT coupling helices along one another to disrupt the interaction with EcfS (Fig. 2f), although other motions of the coupling helices could achieve the same effect¹². Destabilization of the tight interaction between the ECF module and the inward-facing S subunit leads to dissociation of EcfS (Fig. 4b). In the membrane, the released EcfS could reorient towards an outward facing state, which would be driven by the membrane potential due to the positive inside rule for membrane protein topology⁴³. Subsequently, isolated EcfS binds the transport substrate with high affinity^{18,36} (Fig. 4f) with Loop1-2 enclosing the substrate²⁴. Upon association of substrate-bound EcfS and EcfAA'T (Fig. 5 and 6), ATP is hydrolyzed²⁵, leading to opening of the ATPase dimer and an uncharacterized motion of the coupling helices that results in EcfS “toppling” towards the cytoplasm. Subsequently, withdrawal of Loop1-2 from the binding site releases the transport substrate into the cytoplasm. While “toppling” has not been observed, such a dramatic subunit reorientation within the membrane would represent a novel mechanism for substrate translocation.

ONLINE METHODS

Expression and purification of the LmECF–RibU complex

The EcfA'AT operon and the RibU S subunit were amplified from *Listeria monocytogenes* (strain Li 2/ATCC 19115) genomic DNA by PCR. A synthetic operon was constructed by ligating the 3' end Ecf operon to the RibU gene preceded by the following linker sequence and ribosome binding site (underlined): 5' ATAATTTTGTTTAACTTTAAGAAGGAGATATACC 3'. This synthetic operon, with the gene order of EcfA'AT–RibU, was inserted into a modified pET28 vector (EMD4Biosciences) with an amino-terminal decahistidine tag linked by a thrombin recognition site. The LmECF–RibU transporter was expressed in *E. coli* BL21(DE3) by autoinduction in ZYP-5052 media⁴⁴ at 25 °C. After harvesting, cells were resuspended in Buffer A (50 mM Tris, pH 7.5, 500 mM NaCl, 20% glycerol, and 10 mM imidazole) and lysed by cell disruption in an Avestin homogenizer. The membrane fraction was isolated by ultracentrifugation. Membranes were resuspended in 10 ml of Buffer A per gram of membrane and solubilized in 1% dodecyl-maltoside (DDM) for 1 hr at 4 °C with stirring. The LmECF–RibU transporter was bound to TALON resin (Clontech) in DDM and exchanged into SEC buffer (20 mM Tris, pH 7.5, 100 mM NaCl, 10% glycerol, and 0.06% undecyl-maltoside) by thrombin cleavage from washed beads. After removal from the Talon resin, all proteins were treated with 10 mM DTT and 10 mM MgCl₂ and further purified by gel filtration on a Superdex 200 column equilibrated in SEC buffer. For the LmECF–RibU cysteine mutants, 10 mM β-mercaptoethanol was included in the purification buffers prior to gel filtration. Peak fractions were pooled, concentrated in Amicon 100K filtration devices, and were used immediately for assays or flash frozen and stored at –80 °C.

Expression and purification of LmRibU

The gene for LmRibU cloned into a modified pBAD vector (Invitrogen) with a carboxy-terminal myc-decahistidine tag linked by a thrombin recognition site. This construct was transformed into *E. coli* BL21(DE3), and protein expression was performed in LB media at 25 °C. Purification was performed using the same protocol for the entire transporter complex. The purified protein was bound to riboflavin, as evidenced by the yellow color and visible absorbance spectrum of the purified protein^{18,19}. For the LmRibU cysteine mutants, 10 mM β -mercaptoethanol was included in the purification buffers prior to gel filtration.

Expression and purification of His-tagged LmThiT

The gene for the thiamine-binding S subunit from *Listeria monocytogenes*, LmThiT was inserted into a modified pET28 vector with an amino-terminal decahistidine tag linked by a thrombin recognition site. LmThiT was expressed and purified using the same protocol as for LmECF–RibU, except that the protein was eluted from the TALON resin with 50 mM Tris, pH 7.5, 150 mM NaCl, 10% glycerol, 400 mM imidazole, and 0.06% UDM. LmThiT purified as a complex with the thiamine transport substrate, as evidenced by the UV absorption spectrum.

Homology modeling of LmECF–RibU and LmRibU

As the EcfA, A', and T subunits of *L. brevis* and *L. monocytogenes* share 51, 49, and 49% identity, respectively, a homology model of the *Listeria* ECF module was constructed based on the 3.0 Å *L. brevis* crystal structure (PDB ID 4HUQ)¹⁰ using Modeler v9.12 (<http://salilab.org/modeller/>). Similarly, a homology model of LmRibU was constructed based on the crystal structure of RibU from *T. maritima* (N.K.K. and D.N.W, manuscript in preparation), which share 27% sequence identity. Subsequently, Loop1-2 of the LmRibU model was placed into the open conformation. This apo RibU was docked into the LmEcfAA'T model by superposition with the S subunit in 4HUQ. The final model was then energy minimized in PHENIX⁴⁵. The root mean squared deviations (r.m.s.d.) between the model and template structures are 0.3, 0.3, and 1.5 Å for the EcfA, EcfA', and EcfT subunits, respectively.

Cysteine accessibility determined by TMRM labeling of LmRibU and LmECF–RibU

Protein samples were diluted to a final concentration of 10 μ M in degassed SEC buffer and kept on ice. To initiate labeling, 100 μ M tetramethylrhodamine-5-maleimide (TMRM, T-6027, Invitrogen) was added and reactions were incubated on a rocking platform at 4 °C. At selected time points, 15 μ l was removed from the reaction and mixed with 2 μ l of 100 mM DTT to quench the remaining free label. Samples were subsequently mixed with SDS–PAGE sample buffer and electrophoresed on 15 % acrylamide gels. Fluorescent protein bands were visualized on a Typhoon Trio scanner and quantified by ImageQuant v5.2 software (GE Healthcare). The initial rate of TMRM labeling was determined by linear regression of the fluorescence volume of each labeled band over the time points of the assay. All curve fitting and graphs were performed with Prism 5 (Graphpad Software, Inc.).

Crystallization and structure determination of the AMPPNP bound TmEcfAA' heterodimer

The TmEcfAA' heterodimer was expressed and purified as described²⁵. TmEcfA' contained 7 point mutations designed to improve crystal packing. After concentration to ~7 mg/ml, 10 mM MgAMPPNP was added and the sample was incubated on ice for 20 min. Crystals were grown by vapor diffusion from drops consisting of 1.2 μ l protein and 1.2 μ l 100 mM Tris, pH 8.5, 50 % MPD, and 200 mM (NH₄)H₂PO₄ at 4 °C. Crystals were flash frozen in liquid nitrogen prior to data collection in oscillation mode at beamline X29A of the National Synchrotron Light Source. X-ray diffraction data processed with HKL2000⁴⁶. The crystal structure was solved by molecular replacement in Phaser⁴⁷ with PDB entry 4HLU²⁵ as the search model, and refined with PHENIX⁴⁵. No electron density was present for residues 70-71 and 266 of TmEcfA and 101-103 and 250-255 of TmEcfA' and these residues were not included in the final model. The refined model had a Molprobity⁴⁸ score of 2.10. All structure figures were prepared with Pymol (<http://www.pymol.org>).

Reconstitution of LmECF–RibU into liposomes

Purified LmECF–RibU was reconstituted into a 3:1 (w/w) mixture of *E. coli* total lipids (Avanti) and hen egg phosphatidylcholine at a protein to lipid ratio of 1:100 using the protocol described in⁴⁹.

ATPase activity determination of LmECF–RibU

The ATPase activity of the WT and mutant transporter in liposomes was measured in 100 mM NaCl, 0.2 mM EDTA, 50 mM Tris, pH 8.0, with a protein concentration of 0.1 μ M. Reactions with the detergent purified proteins were performed in the same buffer supplemented with 0.06 % UDM and a protein concentration of 0.2 μ M. All reactions were performed at 25 °C. Hydrolyzed phosphate was detected by the Malachite Green assay⁵⁰. Samples lacking enzyme were performed in parallel for background subtraction.

Analytical SEC and multi-angle laser light scattering of ECF transporters

Concentrated protein samples were diluted to desired concentrations in SEC buffer. For the analytical SEC experiments in Figure 4, all proteins were at a final concentration of 5 μ M. Incubation with nucleotides was performed for 10 min at 25 °C using 2 mM concentration of each species. For each sample, 50 μ l was injected on a Shodex KW804 analytical SEC column (Thomson, Clear Brook, VA) equilibrated with 50 mM Tris, pH 7.5, 200 mM Na₂SO₄, 3 mM NaN₃ and 0.05 % DDM on an HPLC (Waters, Milford, MA) equipped with UV and fluorescence detectors. SEC peak positions and heights were determined with the Empower software (Waters). For MALS experiments, 50 μ l of 20 μ M WT or 10 μ M H199'A H204A LmECF–RibU was analyzed as above, except the laser light scattering and refractive index of each sample were measured by a MiniDawn Treos and Optilab Rex, respectively (Wyatt Technology, Santa Barbara, CA). The molecular weight of the LmECF–RibU complexes was calculated using the methodology described in²⁰.

Detection of riboflavin binding by FSEC

Samples were incubated with 20 μ M riboflavin and subject to analytical SEC as described, and the elution position of riboflavin was detected by its intrinsic fluorescence ($\lambda_{\text{ex}} = 440$ nm, $\lambda_{\text{em}} = 520$ nm).

Generation of TMRM-labeled LmRibU (RibU*)

The P34C mutant of LmRibU was expressed and purified as described above. After the TALON purification, the purified protein was degassed and incubated with a 10-fold molar excess of TMRM with stirring for 1 hr at 4 °C. Subsequently, the labeling reaction was terminated by the addition of 10 mM DTT. The sample was concentrated and further purified by gel filtration on a Superdex 200 column equilibrated in SEC buffer. Peak fractions were pooled and dialyzed against SEC buffer to remove any remaining free label from the preparation.

Detection of S subunit exchange and competition by FSEC

For the initial experiments, WT or the H199'A H204A mutant of LmECF–RibU and RibU* were mixed at a concentration of 5 μ M each in SEC buffer supplemented with 5 mM DTT. Next, 2 mM of the respective nucleotide species was added to the protein mixture, and samples were incubated at 25 °C for 10 min. The samples were placed on ice and then promptly injected onto the HPLC under the conditions described above. The position of RibU* was tracked by monitoring the TMRM fluorescence ($\lambda_{\text{ex}} = 520$ nm, $\lambda_{\text{em}} = 580$ nm) versus elution position from the column. To investigate the concentration dependence of exchange, 0.9 μ M LmECF–RibU was mixed with varying concentrations of RibU* in the presence of 2 mM MgATP for 10 min at 25 °C. The reactions were terminated by addition of 10 mM EDTA to stop ATP hydrolysis, and 50 μ l of the sample was analyzed by FSEC. The assay was calibrated by determining the FSEC peak area of RibU* at known protein amounts (Supplementary Fig. 6b-d). The amount of RibU* present in the transporter peak was determined by comparing the FSEC peak area of this peak to the standard curve. This approach assumes that the quantum yield of the TMRM fluorophore attached to RibU* is similar in isolation and when bound to the ECF module. For the ThiT competition experiments, 0.9 μ M LmECF–RibU and 5 μ M RibU* were mixed with varying concentrations of ThiT in the presence of 2 mM MgATP and incubated for 10 min at 25 °C. The amount of RibU* present in the transporter peak was determined as described above.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. TMRM labeling of LmRibU and LmECF-RibU

(a) Ribbon diagram of LmRibU homology model with Loop1-2 colored magenta, Loop3-4 green, and Loop5-6 cyan, and Leu25 colored gray. Residues that were mutated to cysteine are labeled and shown as spheres. (b) Rates of TMRM labeling of LmRibU cysteine mutants in solution. (c) Homology model of LmECF-RibU displaying the EcfT-RibU interface. EcfT is colored yellow and RibU as in (a). (d) Rates of TMRM labeling of purified LmECF-RibU cysteine mutants.

Figure 2. Crystal structure of AMPPNP bound EcfAA' heterodimer

(a) Crystal structure of TmEcfAA' bound to ADP²⁵. (b) Crystal structure of TmEcfAA' bound to AMPPNP. Both structures are displayed in surface representation with the subunits colored as indicated, the Q-helices red, and the bound nucleotides as spheres. (c) Ribbon diagram of the Q-helices in the ADP-bound TmEcfAA' structure. (d) Interaction between the Q-helices in the AMPPNP-bound TmEcfAA' structure. Hydrogen bonds are shown as dashed black lines. (e) Ribbon diagram of the open conformation of the EcfAA' heterodimer from the ECF folate transporter structure¹⁰ with attached T subunit coupling helices CH1-2

and CH3 colored blue and orange, respectively. The interface with the bound S-subunit is represented as a grey ellipse. **(f)** Model of the coupling helices in the ATPase closed conformation based upon the AMPPNP-bound structure of TmEcfAA'.

Figure 3. ATPase activity of LmECF-RibU

(a) Initial rate of ATP hydrolysis by LmECF-RibU. **(b)** ATPase activity of wild type (WT) LmECF-RibU in the presence of potential ATPase inhibitors or with a catalytic mutant. The PL samples were measured in proteoliposomes. **(c)** Conformation of the essential catalytic residue His-189 in the EcfA' active site of the AMPPNP-bound crystal structure. The Mg²⁺ cofactor and the pre-hydrolytic water molecule are shown as green and red spheres, respectively. The data in **(a)** and **(b)** represents the mean and standard deviation of 4 (n=4) and 6 (n=6) technical replicates, respectively.

Figure 4. Analytical SEC of WT and hydrolysis deficient mutant of LmECF-RibU
(a) Size exclusion chromatography (SEC) profiles of purified LmRibU, TmEcfAA', and WT LmECF-RibU after incubation in the presence of different nucleotides. **(b)** SEC profiles of the H199'A H204A mutant of LmECF-RibU incubated in the presence of different nucleotides. **(c)** Chromatograms of H199'A H204A LmECF-RibU after incubation over a range of MgATP concentrations. **(d)** RibU peak area from (c) measured against MgATP concentration. **(e)** Chromatograms of H199'A H204A mutant incubated with the transport substrate, riboflavin (vitamin B2) with or without MgATP measured by UV absorbance, **(e)** and riboflavin fluorescence, **(f)**.

Figure 5. ATP-dependent S subunit exchange by LmECF

SEC profiles of LmECF-RibU, TMRM-labeled LmRibU (RibU*), and an equimolar mixture of both proteins incubated in the presence of different nucleotides measured by UV absorbance, **(a)** and TMRM fluorescence, **(b)**. **(c)** SDS-PAGE of LmECF-RibU, RibU*, and the fluorescent SEC peak from **(b)** visualized by Coomassie staining (left) and TMRM fluorescence (right). **(d)** Dependence of S subunit exchange on RibU* concentration. **(e)** Dependence of S subunit exchange on ThiT concentration. **(f)** SDS-PAGE (left) and Western blot (right) of LmECF-RibU, ThiT, and the fluorescent SEC peak corresponding to the (red) experiment in **(e)**. The data in **(d)** and **(e)** represents the mean and standard deviation of 3 ($n=3$) technical replicates. The complete data for **(c)** and **(f)** can be found in Supplementary Data Set 1.

Figure 6. The ATP-bound state of LmECF exchanges S subunits

(a) SEC profiles of H199'A H204A LmECF-RibU (H2A), RibU*, and an equimolar mixture of both proteins incubated in the presence of different nucleotides measured by TMRM fluorescence. (b) Dependence of S subunit exchange by H199'A H204A LmECF-RibU on RibU* concentration. These data represent the mean and standard deviation of 3 (n=3) technical replicates.

Figure 7. Proposed mechanism of group II ECF transporters

ATP binding to the empty, toppled transporter closes the EcfAA' heterodimer sliding the EcfT coupling helices apart. This conformational change releases EcfS, which assumes an extracellular facing orientation in the membrane. Upon transport substrate binding, EcfS associates with the ECF module. Subsequently, ATP hydrolysis opens the ATPase dimer, potentially leading to toppling of EcfS and release of substrate into the cytoplasm.

Table 1
Data collection and refinement statistics (molecular replacement)

TmEcFAA/MgAMPPNP	
Data collection	
Space group	P4 ₂ 1 ₂
Cell dimensions	
<i>a</i> , <i>b</i> , <i>c</i> (Å)	107.28, 107.28, 96.76
α , β , γ (°)	90, 90, 90
Resolution (Å)	25.2-3.0 (3.14-3.0)*
<i>R</i> _{sym}	0.084 (0.82)
<i>I</i> / σ <i>I</i>	33.1 (2.67)
Completeness (%)	93.0 (80.0)
Redundancy	23.8 (23.6)
Refinement	
Resolution (Å)	3.0
No. reflections	10892
<i>R</i> _{work} / <i>R</i> _{free}	0.210/0.267
No. atoms	
Protein	4009
Ligand/ion	64
Water	20
<i>B</i> -factors (Å ²)	
Protein	88.3
Ligand/ion	75.0
Water	92.1
R.m.s. deviations	
Bond lengths (Å)	0.006
Bond angles (°)	1.01

* Values in parentheses are for highest-resolution shell. Data were collected from a single crystal.