Structures of FolT in substrate-bound and substrate-released conformations reveal a gating mechanism for ECF transporters

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Energy-coupling factor (ECF) transporters are a new family of ABC transporters that consist of four subunits, two cytoplasmic ATPases EcfA and EcfA' and two transmembrane proteins namely EcfS for substrate-specific binding and EcfT for energy coupling. Here, we report the 3.2-Å resolution crystal structure of the EcfS protein of a folate ECF transporter from Enterococcus faecalis-EfFolT, a close homologue of FolT from Lactobacillus brevis-LbFolT. Structural and biochemical analyses reveal the residues constituting the folate-binding pocket and determining the substrate-binding specificity. Structural comparison of the folate-bound EfFolT with the folate-free LbFolT contained in the holotransporter complex discloses significant conformational change at the L1 loop, and reveals a gating mechanism of ECF transporters in which the L1 loop of EcfS acts as a gate in the substrate binding and release.

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Energycoupling factor (ECF) transporters were first discovered in bacteria in 1970s; however, the molecular identities of this new family of ATP-binding cassette (ABC) transporters were not known until recent years. Harnessing the energy of ATP hydrolysis, ECF transporters are responsible for micronutrient uptake from the environment. ECF transporters are composed of four subunits, two cytoplasmic ATPases EcfA and EcfA’, and two transmembrane proteins namely EcfS for substrate recognition and EcfT for energy coupling. The most striking divergence, setting ECF transporters apart from the canonical ABC importers, lies in the substrate-binding proteins; the former utilize integral membrane proteins (EcfSs) for substrate binding, while the latter employ periplasmic solute-binding proteins to capture substrate. The EcfT, EcfA and EcfA’ proteins are termed the ECF module. Based on the features of ECF module, two groups of ECF transporters have been distinguished; in group I, each EcfS associates with a dedicated ECF module, while in group II, up to 12 EcfSs could share a common set of ECF modules.

Because of the unique features of ECF transporters, a number of studies have been carried out to elucidate the underlying molecular basis of substrate specificity and transport mechanism. Structures of four different EcfS proteins in substrate-bound form have been solved, which include the riboflavin-specific RibU from Staphylococcus aureus, the thiamin-specific ThiT and biotin-specific BioY from Lactococcus lactis, and the nickel-specific NikM2 from Thermoanaerobacter tengcongensis. These structures addressed the substrate-binding specificity of ECF transporters. Later, the structures of folate and hydroxymethylpyrimidine (the substrate specificity of this transporter is probably related to pyridoxine, and to folate and hydroxymethylpyrimidine ECF transporters) provided an overview of the architecture of the ECF transporter complex. These structures of EcfS proteins (RMSD of 0.7–1.2 Å) which consist of six transmembrane helices (SM1-6, transmembrane helices 1–6) and a substrate pocket are homogenous among different EcfS structures from different species. These structures were used to study the structural elements involved in energy coupling between EcfT-EcfS and EcfA-EcfA’ were disclosed, which are quite different from those of canonical ABC transporters. Recently, the structural basis of ECF module sharing among different EcfS proteins of group II ECF transporters was proposed by comparing the structures of pantothenate, folate and hydroxymethylpyrimidine ECF transporters from Lactobacillus brevis implemented with mutational analyses—different EcfS proteins use a common hydrophobic interaction surface composed of transmembrane helices 1, 2 and 6 to interact with the same EcfT or ECF module. These structural observations are consistent with the results of functional analyses, and have further been used to explain events that occur during the transport process of ECF transporters.

Although a number of structures of individual substrate-binding proteins and holotransporter complexes are available, the mechanism of the large group of ECF transporters has not been elucidated. Of the currently available structures, four individual S-component structures are substrate-bound, and another three different S-component structures contained in the holotransporters are substrate-free. However, structures of a single S component crystallized in both states are lacking. Here we report the structure of the EcfS protein of a folate ECF transporter from Enterococcus faecalis-EfOIT in complex with its substrate folate. Structural and biochemical analyses reveal the residues responsible for folate-specific binding. More importantly, structural comparison of the folate-bound EfOIT with the previously reported structure of folate-free FolT from L. brevis-LbFolT contained in the holotransporter complex reveals a gating mechanism of ECF transporters.

Results
Structure determination of substrate-bound EfOIT. To study the underlying mechanism of substrate binding and release from ECF transporters, we focused on the crystallization of the EcfS proteins of folate ECF transporters-FotT. Several FotT proteins from different species were homogenously purified, but only the FotT protein from E. faecalis (EfOIT) could be successfully crystallized. Numerous efforts were made to optimize the crystals, including the addition of 10-fold excess (molar ratio) amount of folate in the purified protein solution, which was found to be essential for the growth of diffractable crystals. In the end, an anisotropic X-ray data set (2.7–3.5 Å) was collected by screening hundreds of crystals. The data were truncated to 3.2-Å resolution based on the analyses of the UCLA-MBI diffraction anisotropy server. The structure of EfOIT was determined with molecular replacement method using the substrate-free LbFotT (Lb. L. brevis) structure as a template. There are six EfOIT molecules in one asymmetric unit. The electron density for molecules A, B, C and D are quite good for modelling, while the density for molecules E and F are poor and the models are built based on molecule A structure (Supplementary Fig. 1). The statistics of data collection and structure refinement are summarized in Supplementary Table 1.

There are small conformational differences among the six molecules (root mean square deviations/RMSD of 0.7–1.2 Å). The overall structure of EfOIT is similar to the reported structures of EcfS proteins (RMSD of 1.5–3.3 Å) which consist of six transmembrane helices (SM1-6, transmembrane helices 1–6 of EcfS) that form a helix bundle. The N-terminus and C-terminus are both at the cytoplasmic side. Three loops L1, L3, L5 connect SM1-SM2, SM3-SM4 and SM5-SM6, respectively, from the periplasmic side (Fig. 1a). A pocket with the depth of 24 Å and volume of 1,230 Å³ is formed by the six transmembrane helices near the periplasmic side and is covered by the L1 loop from top (Fig. 1b). The substrate bound in the pocket is verified by mass spectrometry to be folate (Fig. 1c) and further confirmed by the electron density map in the pocket (Fig. 2a).

The folate-binding site. The folate molecule adopts an ‘L’-shaped conformation, with the pterin moiety forming the short arm and the aminobenzoate and glutamate moieties forming the long arm (Fig. 1a,b). A similar conformation of folate has been found in the human folate receptor FR. The recognition of folate is mainly through the pterin and glutamate moieties by a number of residues protruding from SM1, L1 loop and SM3-6 (Fig. 2c). Specifically, the pterin ring of folate forms π-π interactions with the parallel side chain of Phe80. The pterin N2 atom forms two hydrogen bonds with the guanidine group of Arg26 (Fig. 2d). The glutamate moiety of folate is stabilized mainly through six hydrogen bonds. Specifically, the O1 and O2 atoms of glutamate group form four hydrogen bonds with the side chains of residues Asn117, Thr121 and Arg142; and the OE1 and OE2 atoms form two hydrogen bonds with the side chains of Lys36 and Lys145. In addition, Arg142 forms another hydrogen-bonding interaction with the carbonyl oxygen of aminobenzoate moiety (Fig. 2e). The extensive interactions between folate and EfOIT are consistent with the high binding affinity determined by Isothermal Titration Calorimetry (ITC)
experiments (dissociation constant/$K_D$ of 29.8 nM for folate) (Fig. 3), and the previously reported high binding affinity of $Lc$FolT ($Lc$, *L. casei*) with folate.$^{23}$ In addition, the above residues involving folate binding are highly conserved, suggesting that a common folate recognition mode could exist among the FolT proteins of ECF transporters (Supplementary Fig. 2).

Figure 1 | Overall structure of $Ef$FolT. (a) Overall structure of $Ef$FolT in ribbon cartoon. The six transmembrane helices 1–6 (SM1–6) are coloured from blue to red. The bound substrate folate is shown with a stick model coloured in magenta. (b) A cross-section drawing of $Ef$FolT shows the folate-binding pocket (blue and red colours represent positive and negative charges, respectively). (c) Mass spectrometry results of the substrate bound with $Ef$FolT. Lower panel shows the folate standard; upper panel shows the substrate bound with $Ef$FolT.

Figure 2 | Folate-binding site of $Ef$FolT. (a) Electron density of folate at the substrate-binding pocket of $Ef$FolT (Fo–Fc density contoured at 2.0 σ level in molecule A). Folate is shown with a ball-and-stick model in magenta. (b) Chemical structure of folate. (c) Top view of the folate-binding pocket of $Ef$FolT. Structure of $Ef$FolT is shown with light-blue ribbons, and residues that line the pocket are shown with yellow sticks. (d, e) Close-up views of the interactions of pterin moiety (d), and glutamate and aminobenoate moieties (e) of folate with surrounding residues. The hydrogen bonds are indicated by dashed lines.
Asp64; Arg142; Phe80

Folate binding can be ranked according to the above results: (Table 1). Taken together, the contributions of these residues to the carboxyl group of folate is critical for the folate binding bond formed between the residue Lys/Arg36 from the L1 loop, it is conserved but is replaced with Arg in some species; whereas the folate-binding affinity of R142A or K145A/T mutants is decreased by 10-fold and 110-fold respectively, whereas the folate-binding affinity of N117A mutant is slightly decreased, and that of K36A is significantly reduced by a factor of 180 while K36D mutant loses the folate-binding affinity, which suggests that the hydrogen bond formed between the residue Lys/Arg36 from the L1 loop and the carboxyl group of folate is critical for the folate binding (Table 1). Taken together, the contributions of these residues to folate binding can be ranked according to the above results: Asp64; Arg142; Phe80; Lys117; Thr81; Arg26; and Thr121. A significant difference is observed at the L1 loop. In EfFoT, the L1 loop covers on the substrate-binding pocket mutants assayed by isothermal titration calorimetry.

Table 1 | Folate-binding affinity of EfFoT substrate-binding pocket mutants assayed by isothermal titration calorimetry.

| Mutants | Dissociation constant/Kd (nM) |
|---------|-----------------------------|
| WT      | 29.8 ± 4.7                  |
| R26A    | 251.9 ± 22.0                |
| D64A    | No binding*                 |
| T81A    | 813.5 ± 162.1               |
| F80A    | No binding                  |
| F80W    | 498.8 ± 25.8                |
| N117A   | 68.6 ± 6.4                  |
| T121A   | 288.1 ± 21.1                |
| R142A   | No binding                  |
| K145A   | 330 ± 198                   |
| K145T   | 3,690 ± 222                 |
| K145D   | No binding                  |
| K36A    | 5,403 ± 549                 |
| K36R    | 91.5 ± 7.5                  |
| K36D    | No binding                  |
| F34A    | 68.4 ± 4.7                  |
| Y129A   | 634.7 ± 33.6                |
| W138A   | 147.3 ± 15.0                |

Conformational change of FolT induced by folate binding. The two EcfS proteins of folate ECF transporters, EfFoT and LbFoT share sequence similarity of 54.9% and identity of 33.0%, and the structure of EfFoT represents the folate-binding state conformation, while the structure of LbFoT contained in the folate ECF transporter complex represents the folate-released state conformation; therefore, we compare these two structures to investigate the underlying mechanism of substrate binding and release. Superimposition of the EfFoT and LbFoT structures reveals a RMSD of 1.5 Å, and significant conformational difference is observed at the L1 loop. In EfFoT, the L1 loop covers on the substrate-binding pocket and adopts a ‘closed’ conformation, while in LbFoT, the same loop flips away from the pocket and adopts an ‘open’ conformation (Fig. 4a,b). Based on this data, we suggest that folate binding can induce a conformational change at the L1 loop. In the folate-released or free state, the L1 loop adopts an ‘open’ conformation to leave the pocket widely open as represented by the LbFoT structure (Fig. 4c); once folate binds into the pocket, residue Arg26 from the C terminal of SM1, and residue Lys36 from the L1 loop form three hydrogen bonds with folate, which we propose pulls the L1 loop from the ‘open’ to the ‘closed’ conformation as represented by the EfFoT structure (Fig. 4b,d). In addition, residues (i.e., residues Phe34 and Leu35) from the L1 loop stack against the residues constituting the L5 loop (i.e., residues Leu125, Tyr129 and Trp138), which may stabilize the ‘closed’ conformation of L1 loop in EfFoT (Fig. 4d). Mutation of these residues can decrease folate binding (Table 1 and Supplementary Fig. 5). These observations suggest that the L1 loop acts as a gate in controlling the substrate binding and release of the folate ECF transporter.

Discussion

In this work, the structure of the EcfS protein of a folate ECF transporter-EfFoT is determined in the folate-bound conformation which revealed key residues responsible for substrate-specific binding. For the first time, the substrate-binding-induced conformational changes of the EcfS protein of ECF transporters can be clearly seen by comparing the structures of substrate-bound EfFoT with substrate-free LbFoT in the folate ECF transporter complex captured in inward-open conformation. Based on the data presented here, we suggest that the L1 loop acts as a gate in folate binding and release and that folate binding
induces the conformation of the gate to change from ‘open’ to ‘closed’. Our data is supported by a previous report suggesting that the L1 loop of ThiT undergoes conformational changes upon thiamine binding.24 These results raise another important question during the transport process: what drives the change of the L1 loop from the ‘closed’ conformation to the ‘open’ conformation to release the substrate? To provide insights of this question, we modelled the folate-bound $\text{Ef}_{\text{FolT}}$ structure to the quaternary folate ECF transporter complex $\text{Lb}_{\text{ECF-FolT}}$ captured in inward-open state, and extensive interactions are observed between the transmembrane helices 1, 2, 6 of $\text{Ef}_{\text{FolT}}$ and coupling helices 2/3 of $\text{EcfT}$ protein, which are commonly observed among group II ECF transporters.20 In addition, clashes are observed between the L1 loop of $\text{Ef}_{\text{FolT}}$ and the transmembrane helix 3 of $\text{Lb}_{\text{EcfT}}$ (Supplementary Fig. 6). These observations indicate that the driving force is closely related with $\text{EcfT}$. Here, we postulate that the force(s) driving the conformational change of L1 loop come from the relative movement of $\text{EcfS}$ against $\text{EcfT}$ during the transport process (Supplementary Fig. 7): energy coupling helices 2/3 (CH2/3) and probably transmembrane helices of $\text{EcfT}$ undergo conformational changes following the ATP hydrolysis in $\text{EcfA/A’}$ proteins, which may further lead to the changes of transmembrane helices of $\text{EcfS}$, especially SM1 and SM6. As small conformational changes in SM1, SM2, SM6 or L1 loop can affect the residues essential for substrate binding (Figs 2 and 3), i.e., Arg26 from SM1, Lys36 from L1 loop and Arg142 and Lys145 from SM6 in $\text{Ef}_{\text{FolT}}$, the interaction of folate with these residues, especially residues Arg26 and Lys36 which adopt different conformations in $\text{Lb}_{\text{FolT}}$ and $\text{Ef}_{\text{FolT}}$ could initially be broken. As a result, breaking of these interactions will greatly lower the binding affinity of folate with $\text{FolT}$ and speed up the conformational change of L1 loop from ‘closed’ to ‘open’, which finally leads to substrate release (evidenced by the fact that the substrate folate cannot bind with the inward-open folate ECF transporter complex $\text{Lb}_{\text{FolT}}$ as detected by ITC (Supplementary Fig. 5)). Similar mechanism might exist among other ECF transporters as there are essential residues protruding from the above mentioned structural elements and involving substrate binding. Taken together, our results and analyses suggest a gating mechanism of ECF transporters, in which the L1 loop acts as a gate in substrate binding and release.

We also note a recent study about the gating mechanism of RibU, in which the authors performed molecular dynamic simulation analyses and suggested that the L5 loop could serve as a gate in riboflavin binding and release.25 However, our
structural data of the folate ECF transporter do not support this conclusion. Although we also observed conformational change at the L5 loop, we considered it as the stabilization factor of the L1 loop. To be more critical, as the L5 loop of EcfS is distant from the energy-coupling scaffold EcfT in the transporter complex, if L5 loop acts as a gate, then the driving force of the conformational change is questionable. Nevertheless, we cannot exclude the possibility that ECF transporters use different gating mechanisms for substrate binding and release, which will require further structural analysis to determine.

Methods

Gene cloning and protein purification. The gene encoding the S component of the folate ECF transporter from *E. faecalis*, *EfoT*, was cloned into pET28a vector using NdeI and Xhol (primers used are: for-AAAAACATATGATGACAAAGAA AAAAAATTTGG, and rev- AAAAATCGGTATTGTCTAATTCGATA). A tag of six histidine residues was added at the N-terminus of the EfoT. The plasmid was transformed into *Escherichia coli* BL21 (DE3) and induced by a 0.25 mM β-mercaptoethanol (IPTG) at 30°C of about 2 h. After 14 h at 37°C, the cells were harvested, and homogenized in buffer A (100 mM NaCl, 20 mM Tris-HCl pH 8.0), and lysed using a French press. Cell debris was removed by centrifugation. The supernatant was collected and applied to ultracentrifugation at 150,000g for 1 h. Membrane fraction was incubated with 2% (w/v) n-nonyl-β-D-glucopyranoside (NG; Anatrace) for 2 h at 4°C. After another centrifugation step at 20,000g for 45 min, the supernatant was loaded onto a Ni²⁺-NTA affinity column (Qiagen), and washed with buffer B (100 mM NaCl, 20 mM Tris-HCl pH 8.0 and 0.4% NG) plus 25 mM imidazole. The protein was eluted from the column using buffer B plus 250 mM imidazole, and was concentrated to about 10 mg ml⁻¹ before further purified by gel filtration (Superdex 200, GE Healthcare) in buffer B. The peak fraction was collected and concentrated to ~5 mg ml⁻¹ for crystallization.

Identification of substrate bound with EfoT using liquid chromatography-mass spectrometry. Equivalent volume of acetoneitrile was added to the purified EfoT solution (10 mg ml⁻¹, 500 μl) to denature the protein. Then the sample was sonicated for 15 min at room temperature to release the substrate. After centrifugation at 12,000 r.p.m. for 10 min, the supernatant was filtered through a 0.22-μm membrane before loaded onto the C18 column connected with Agilent G6230B HPLC system. Accurate mass quadrupole time-of-flight liquid chromatography-mass spectrometry system.

Crystalization and structure determination. For crystallization of EfoT, 10-fold molar excess amount of folate was added to the purified protein solution before crystallization. The crystallization was performed at 20°C using sitting drop vapour diffusion method. Small crystals were found in several conditions. For crystallization of *EfFolT-truncate*, a 45 min, the supernatant was loaded onto a Ni²⁺-NTA affinity column (Qiagen), and washed with buffer B (100 mM NaCl, 20 mM Tris-HCl pH 8.0 and 0.4% NG) for 2 h at 4°C. After another centrifugation step at 20,000g for 45 min, the supernatant was loaded onto a Ni²⁺-NTA affinity column (Qiagen), and washed with buffer B (100 mM NaCl, 20 mM Tris-HCl pH 8.0 and 0.4% NG) plus 25 mM imidazole. The protein was eluted from the column using buffer B plus 250 mM imidazole, and was concentrated to about 10 mg ml⁻¹ before further purified by gel filtration (Superdex 200, GE Healthcare) in buffer B. The peak fraction was collected and concentrated to ~5 mg ml⁻¹ for crystallization.

To solve the structure, molecular replacement was used with the structure of FolT of *E. coli* as a template (PDB ID: 4HUQ). Six molecules were found in one asymmetric unit, and four of them (molecules A, B, C, D) have high-quality electron density which allowed the auto-building of the C-terminus of the ECF transporter in *Lactobacillus casei*: evidence for a component shared with the thiamine and biotin transport systems. *J. Bacteriol.* 137, 1308–1314 (1979).

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Author contributions
Q. Z., Chengcheng W. and P.Z. designed the experiments; Q. Z. and Chengcheng W. performed the majority of the experiments; Chengyuan W. carried out the structure determination; all authors contributed to data analysis and manuscript preparation; P.Z. conceived the project and wrote the paper.

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