Chapter 3

Quaternary Structure and Functional Unit of Energy Coupling Factor (ECF)-type ABC Transporters


3.1 Abstract

ATP-binding cassette (ABC) transporters mediate transport of diverse substrates across membranes. We have determined the quaternary structure and functional unit of the recently discovered ECF-type (energy coupling factor) of ABC transporters, which is widespread among prokaryotes. ECF transporters are protein complexes consisting of a conserved energizing module (two peripheral ATPases and the integral membrane protein EcfT) and a non-conserved integral membrane protein responsible for substrate specificity (S-component). S-components for different substrates are often unrelated in amino acid sequence but may associate with
the same energizing module. Here, the energizing module from *Lactococcus lactis* was shown to form stable complexes with each of the eight predicted S-components found in the organism. The quaternary structures of three of these complexes were determined by light scattering. EcfT, the two ATPases (EcfA and EcfA’), and the S-components were found to be present in a 1:1:1:1 ratio. The complexes were reconstituted in proteoliposomes and shown to mediate ATP-dependent transport. ECF-type transporters are the smallest known ABC transporters.

### 3.2 Introduction

ATP-binding cassette (ABC) transporters catalyze the transport of a wide variety of molecules across lipid bilayers into or out of cells and organelles. They form one of the largest known protein families and are found in organisms from all kingdoms of life (Higgins, 1992). ABC transporters consist of two structurally related or identical integral membrane domains that form the translocation pore and two peripheral nucleotide-binding domains (ATPases) that energize the transport. ABC importers are found only in prokaryotes and may require additional extracellular or periplasmic soluble substrate-binding domains or proteins (Higgins, 1992; Rees et al., 2009; Davidson et al., 2008). Recently, a new type of ABC importer was found, which is widespread among prokaryotes and was named the ECF-type (for energy coupling factor) (Hebbeln et al., 2007; D. A. Rodionov et al., 2008). Bioinformatics analyses have predicted that ECF-type ABC transporters are involved in the uptake of vitamins or other nutrients needed in trace amounts (such as Ni$^{2+}$ or Co$^{2+}$ ions) (D. Rodionov et al., 2009). They consist of peripheral (ATPase) and transmembrane subunits that together form an energizing module. ECF-type transporters do not make use of soluble substrate binding proteins, but employ integral membrane proteins (named S-components) to bind their substrates. The S-components are small (20 - 25 kDa) and hydrophobic (4 - 6 predicted transmembrane segments) (D. A. Rodionov et al., 2008). The crystal structures of three S-components are now available: RibU from *Staphylococcus aureus* (Zhang et al., 2010), and the structures of ThiT (Erkens et al., 2011) and BioY (see chapter 4) both from *Lactococcus lactis*. The crystallized S-components bind different substrates (riboflavin, thiamin and biotin, respectively) and are unrelated in amino acid sequence, but they have the same fold. This fold is not related to the membrane domains of other ABC transporters (Zhang et al., 2010).
ECF-type ABC transporters fall into two groups based on their genomic organization (D. A. Rodionov et al., 2008). In the first group, each S-component is encoded in the same operon as an energizing module (hence designated as dedicated energizing modules). The biotin transporter BiMN from *Rhodobacter capsulatus* is the best-characterized member of this group (Hebbeln et al., 2007). ECF transporters of the other group consist of energizing modules that are encoded in an operon without S-component genes. These energizing modules usually consist of two different homologous ATPases (EcfA and EcfA’), alongside the membrane protein EcfT, and are predicted to interact with various S-components that are encoded by genes scattered over the genome (hence the name shared energizing modules) (D. A. Rodionov et al., 2008). The different S-components are often unrelated at the sequence level. The shared ECF transporters are particularly abundant in Gram-positive organisms, many of which are pathogens.

Here, we present the first genome-wide experimental analysis of an ECF-type transporter with a shared energizing module. We reveal the quaternary structure and minimal functional unit using purified proteins solubilized in detergent or reconstituted in proteoliposomes.

### 3.3 Materials and Methods

#### 3.3.1 Cloning and Expression of EcfAA’T and EcfAA’T-S-component Complexes.

The *ecf* operon from *Lactococcus lactis* (consisting of the genes annotated as *cbiO*, *cbiO*, and *cbiQ*2, here renamed as *ecfA*, *ecfA’*, and *ecfT*) was cloned in a pNZ8048 vector (Ruyter et al., 1996) for expression in the *L. lactis* strain NZ9000 (Kuipers et al., 1998) and in a pBAD vector (Guzman et al., 1995; Geertsma & Poolman, 2007) for expression in the *Escherichia coli* strain MC1061 (Wertman et al., 1986). The sequence coding for a His-tag was added in-frame at the 5’ end of the *ecfT* gene (*ecfT*-His) or the 3’ end of the first *cbiO* (His-ecfA) gene via the ligation independent cloning method (Geertsma & Poolman, 2007). In between the 10-His tag and the gene is a TEV cleavage site. For simultaneous expression of the *ecf* operon and the genes encoding S-components in *E. coli* MC1061, an expression vector based on pBAD24 was used called p2BAD (see figure 3.1) (Birkner et al., 2012). This vector contains a pBR322 origin of replication, an ampicillin resistance marker gene
and two arabinose-inducible promoters (pBAD), both with a downstream multiple cloning site and a terminator region (Birkner et al., 2012). In a first step the gene for the S-component of interest was placed with a STREPII-tag (WSHPQFEK) behind the second promoter of the pBAD vector, via XbaI and EcoRI or XhoI restriction sites. Then, the ecfAA’T genes were placed behind the first promoter. This was done by removing part of the pBAD promoter from the p2BAD plasmid and replacing it with part of the pBAD promoter with ecfAA’T from the pBAD:ecfAA’T plasmid that was constructed via ligation independent cloning LIC (Geertsma & Poolman, 2007). p2BAD(-,s-component) and pBAD(ecfAA’T) were both cut with BspE1 and HindIII or BglII. The right fragment of p2BAD was isolated from gel, while for pBAD the right fragment was isolated by simply cutting the other fragment to pieces by using additional restriction enzymes (that did not cut in the ecfAA’T containing fragment). The desired fragments (one containing part of the promoter with ecfAA’T from pBAD and the other containing the p2BAD with only a part of the first promoter missing until the first multiple cloning site) were then ligated with T4 ligase (NEB), for ~1 hour at 16°C. In this way the ecfAA’T could be transferred from the pBAD into a p2BAD vector.

Figure 3.1. The p2BAD vector used for co-expression of the EcfAA’T-10His and an S-component (in this case RibU) in E. coli. The ecfAA’T operon is under control of the first arabinose promoter and an S-components (in this case ribU) is under control of the second arabinose promoter. Only the restriction sites discussed in the text are indicated.

*L. lactis* cells were cultivated semi-anaerobically at 30 °C in either M17 (Terzaghi & Sandine, 1975) (used for small-scale cultures) or a medium containing 2% (w/v) gistex LS (Strik BV, Eemnes, NL) and 65 mM sodium phosphate, pH 6.5 (used for large-scale cultures in the bioreactor). Both media were supplemented with 1% (w/v) glucose and, when pNZ8048 derived plasmids were present, 5 µg/mL chloramphenicol. For large-scale protein production *L. lactis* was grown in a 10 liter batch reactor (Applikon Biotechnology, Delft) at 30°C and pH 6.5 (adjusted by KOH addition), stirred at 300 rpm. Expression was induced with 0.1% (v/v) culture supernatant of the nisin A producing strain NZ9700 when the cells reached an optical density (OD) of 2 measured at 600nm (Kunji et al., 2003). *E. coli* was cultivated using 2xTY medium (16 g/L bactoTM tryptone, 10 g/L BBLTM yeast extract and 5 g/L NaCl) for large cultures in the bioreactor or LB for all other cultures. Small-scale cultures in Erlenmeyer flasks were incubated at 37 °C and shaken at 200 rpm. For large-scale cultures a 2 liter batch reactor (Applikon Biotechnology, Delft) was used and operated at 37°C, pH 7.5, 60% air (flow console, Applikon Biotechnology, Delft) and stirred at 800 rpm. When the cells had reached OD~1.8 (at 600 nm) the rotor speed was raised to 1000 rpm and the temperature was lowered to 25 °C. Once this temperature was reached (at OD~2), 10−3% L-arabinose was added. When the cells stopped growing 0.2% glycerol was added to obtain a higher biomass. Both *L. lactis* and *E. coli* cells were harvested after 3 hours of induction by centrifugation at 9000 xg, 20 min, 4 °C followed by resuspension in 50 mM potassium phosphate, pH 7.5, to OD~200. The cell suspension was flash frozen in liquid nitrogen and stored at -80 °C.

3.3.3 Preparation of membrane vesicles.

The frozen cells were thawed at room temperature, diluted to OD~100 with ice-cold potassium phosphate buffer supplemented with 1 mM PMSF, 1 mM MgSO₄ and DNase (~50 µg/mL). Cells were broken using a cell disrupter operated at 5 °C (Constant Systems Ltd). *E. coli* cells were passed once through the disrupter (at 25 kPsi) whereas *L. lactis* cells were passed twice (at 39 kPsi). Cell debris was removed by centrifugation (20 min, 118000 xg at 4 °C) and subsequently the membrane vesicles were collected by ultracentrifugation (60 min, 185000 xg at 4
The membrane vesicles were kept on ice and resuspended in 50 mM potassium phosphate, pH 7.5, and the total protein content was measured with a BCA protein assay (Thermo Scientific Pierce). Finally, they were aliquotted, flash frozen in liquid nitrogen and stored at -80 °C.

3.3.4 Protein purification.

Membrane vesicles were solubilized at a total protein concentration of 5 mg/mL in a buffer containing 50 mM potassium phosphate, 10% glycerol, 300 mM NaCl, 15 mM imidazol and 0.5% n-dodecyl-β-d-maltoside (DDM), pH 7.5. The solution was kept on ice for 1 h with occasional rocking. Unsolubilized material was removed by centrifugation (20 min, 327578 xg, 4 °C) and the supernatant was mixed with 0.5 mL Nickel-Sepharose chromatography material (GE-Healthcare) equilibrated in solubilization buffer without DDM. The suspension was incubated for 1 h on a rotating platform at 4 °C, transferred to a disposable column (Biorad) and the column material was allowed to settle. Subsequently, the column was washed with 20 column volumes of wash buffer (50 mM potassium phosphate, 10% glycerol, 300 mM NaCl, 50 mM imidazol and 0.05% DDM, pH 7.5). The proteins were eluted in three fractions of 350 µL, 650 µL and 500 µL respectively using elution buffer (50 mM potassium phosphate, 10% glycerol, 300 mM NaCl, 500 mM imidazol and 0.05% DDM, pH 8). The second elution fraction was centrifuged at 20800 xg and 4 °C to remove possible aggregates/particles. 600 µL of the supernatant was loaded on a 23 mL Superdex 200 gel filtration column (GE Healthcare), equilibrated with gelfiltration buffer (50 mM potassium phosphate, pH 7.5, 150 mM NaCl and 0.03% DDM) at 4 °C. After the size-exclusion run, fractions containing the ECF-complexes were collected, placed on ice and used immediately for reconstitution in proteoliposomes or light scattering experiments.

3.3.5 Light Scattering.

The subunit stoichiometries of the complexes were determined by size-exclusion chromatography coupled to multi-angle laser light scattering (SEC-MALLS) (Slotboom et al., 2008; Wen et al., 1996). SEC-MALLS was performed as described before (Erkens & Slotboom, 2010; Slotboom et al., 2008). 200 µL of the purified protein was used in the experiment (~155 µg). Note that the size exclusion column is used only for the separation of protein-detergent-micelles from detergent-only-
micelles; the molecular mass of the protein component of the protein-detergent-micelle is calculated precisely based on the absorbance at 280 nm, the refractive index and the light scattering. The elution volume is not used for the determination. For calculation of the molecular mass of multisubunit complexes of membrane proteins, we made use of the internal consistency method described by Wen et al. (Wen et al., 1996). This method assumes several different possible subunit stoichiometries and then determines the molecular mass from the light scattering data, which should be consistent with the stoichiometry initially assumed.

3.3.6 Analysis via SDS-PAGE and/or Western blotting.

To 40 µL of protein samples, 10 µL 5x SDS-PAGE loading buffer was added (120 mM Tris-HCl pH 6.8, 50% glycerol, 100 mM DTT, 2% (w/v) SDS, and 0.1% (w/v) bromophenol blue). The samples were then analyzed on a 12% SDS-polyacrylamide gel. Subsequently these gels were either stained with Coomassie or transferred to a PVDF membrane via semidry electroblotting. Immunodetection of proteins on the Western blots was done with a primary antibody raised against a His6-tag (Amer-sham Pharmacia Biotech) or against the STREPII-tag (Qiagen). Chemiluminescence detection was done by using the Western light kit (Tropix, Inc.) and the LAS-3000 imaging system (Fujifilm) with AIDA software (Raytest).

3.3.7 Analysis via MS/MS.

For mass spectrometry (tandem MALDI-TOF) analysis, all the visible bands on a Coomassie Brilliant Blue-stained gel were excised. The gel bands were destained and digested overnight with trypsin (Promega V5111), and peptides were extracted as described by Kiel et al. (Kiel et al., 2009). The peptide digests were mixed 1:1 (v/v) with a solution of α-cyano-4-hydroxycinnamic acid matrix (5 mg/ml in 50% acenotrile and 0.1% TFA, LaserBio Labs), spotted onto a stainless steel MALDI target, and analyzed with a 4700 proteomics analyzer MALDI-TOF/TOF mass spectrometer (Applied Biosystems).

3.3.8 Reconstitution and Transport Assays in Proteoliposomes.

The purified complexes EcfAAT-NiaX and EcfAAT-RibU were reconstituted into proteoliposomes, using the previously described method (Geertsm et al., 2008).
The ECF complexes were reconstituted into liposomes composed of *E. coli* polar lipids and egg phosphatidylcholine (3:1, w/w) at protein-to-lipid ratios (w/w) of 1:250 (EcfAAT-NiaX for uptake experiments), 1:100 (EcfAAT-NiaX for efflux experiments), and 1:333 (EcfAAT-RibU for uptake experiments). For use in the transport assays, proteoliposomes were thawed, and the solute composition was adjusted to match the desired luminal composition: 50 mM potassium phosphate, with 10 mM ATP and 10 mM MgSO$_4$, 10 mM ADP and 10 mM MgSO$_4$, 10 mM MgSO$_4$ only, or 10 mM AMP-PNP with 10 mM MgSO$_4$. In all cases, the pH was 7. Subsequently, the suspension was frozen in liquid nitrogen and thawed three times. Subsequently, the proteoliposomes were extruded 11 times through a 200 nm pore size polycarbonate filter (Avestin) and centrifuged (267,000 xg, 20 min, 4°C). For the transport assays, the proteoliposomes were diluted to an estimated protein concentration of 5 µg/mL. 200 µL aliquots were made, one for each time point, and transport was started by the addition of radiolabeled substrate. In the case of EcfAAT-NiaX, [³H]niacin was added to a final concentration of 375 nM, and in the case of EcfAAT-RibU, 35 nM [³H]riboflavin was used. At the indicated time points, 2 mL of stop buffer (ice-cold 80 mM potassium phosphate, pH 7) was added. Once the stop buffer had been added, the solution was rapidly filtered over a BA-85 nitrocellulose filter, which was subsequently washed once with 2 mL of stop buffer. Filters were dried for 1 h at 80 °C, 2 mL of Emulsifier-Scintillator Plus liquid (PerkinElmer Life Sciences) was added, the suspension was vortexed, and levels of radioactivity were determined with a PerkinElmer Tri-Carb 2800 TR isotope counter.

### 3.3.9 Efflux assay.

For the efflux experiments EcfAAT-NiaX proteoliposomes were freeze-thawed (using liquid nitrogen) eight times instead of three times in order to scramble the orientation (Geertsma et al., 2008). Proteoliposomes were diluted to 0.7 µg/µL protein in 50 mM potassium phosphate, pH 7, and incubated with 10.1 µM [³H]niacin for four hours at 6 °C. Then the suspension was further diluted in the same buffer without radiolabeled substrate to 25 µg/mL protein and incubated at 25 °C. At the indicated time points, 100 µL aliquots were taken, filtered, washed and dried as described above. After 3 minutes either 9.1 mM Mg-ATP was added or -as a control- the same volume of buffer.
3.4 Results

The genome of the Gram-positive bacterium *L. lactis* contains one operon coding for a shared energizing module (*ecfAA’T*, annotated as *cbiOOQ*). In addition, it contains eight different genes coding for predicted S-components with confirmed or predicted specificity for vitamins and their precursors (Erkens & Slotboom, 2010; Duurkens et al., 2007; D. Rodionov et al., 2009) (Table 3.1).

To investigate the quaternary structure of the ECF-type ABC transporters, we initially aimed to isolate the energizing module EcfAA’T from *L. lactis* (Kunji et al., 2003). His-tagged EcfAA’T was produced in *L. lactis*, solubilized, and purified by nickel affinity and size-exclusion chromatography. Figure 3.2a shows an SDS-PAGE analysis of the purified complex. Besides the three proteins from the energizing module (His-EcfA, EcfA’, and EcfT), we consistently co-purified several proteins with molecular masses around 20 kDa. We hypothesized that these proteins could be the endogenous S-components from *L. lactis* that had formed stable complexes with the energizing modules. Indeed, one of the proteins (indicated in figure 3.2a) was identified by MALDI mass spectrometry as the S-component NiaX. We could not reveal the identities of the other co-purified proteins, presumably because the physicochemical properties of the S-components (small and very hydrophobic proteins) precluded identification by in-gel protein digestion, peptide extraction, and mass spectrometry.

Table 3.1. S-components found in *L. lactis* and predicted to interact with the shared energizing module (D. Rodionov et al., 2009). a.a., amino acids.

<table>
<thead>
<tr>
<th>Name</th>
<th>(Probable) substrate</th>
<th>Size (a.a.)</th>
<th>Size (kDa)</th>
<th>Predicted number of TM helices</th>
<th>Accession code in genome</th>
</tr>
</thead>
<tbody>
<tr>
<td>BioY</td>
<td>Biotin</td>
<td>189</td>
<td>20.5</td>
<td>6</td>
<td>ilmg_1964</td>
</tr>
<tr>
<td>BioY2</td>
<td>Biotin</td>
<td>182</td>
<td>19.7</td>
<td>6</td>
<td>ilmg_0332</td>
</tr>
<tr>
<td>PdXU2d</td>
<td>Pyridoxine (related)</td>
<td>166</td>
<td>18.1</td>
<td>4 or 5</td>
<td>ilmg_0464</td>
</tr>
<tr>
<td>NiaX</td>
<td>Niacin</td>
<td>222</td>
<td>24.6</td>
<td>5 or 6</td>
<td>ilmg_1330</td>
</tr>
<tr>
<td>PanT</td>
<td>Pantothenic acid</td>
<td>196</td>
<td>21.1</td>
<td>6</td>
<td>ilmg_0542</td>
</tr>
<tr>
<td>QueT</td>
<td>Queuosine precursor</td>
<td>169</td>
<td>19.1</td>
<td>4 or 5</td>
<td>ilmg_1760</td>
</tr>
<tr>
<td>RibU</td>
<td>Riboflavin</td>
<td>206</td>
<td>23.0</td>
<td>5 or 6</td>
<td>ilmg_1195</td>
</tr>
<tr>
<td>ThiT</td>
<td>Thiamine</td>
<td>182</td>
<td>19.9</td>
<td>5 or 6</td>
<td>ilmg_0334</td>
</tr>
</tbody>
</table>

a Substrate specificity has been confirmed experimentally for RibU (Duurkens et al., 2007), ThiT (Erkens & Slotboom, 2010), PanT (chapter 6) and from *Leuconostoc mesenteroides* (Neubauer et al., 2009), NiaX (this chapter), BioY (chapter 4) and BioY2 (chapter 6).

b Protein sequences were analyzed by topcons, which runs five different prediction programs. The number of predicted transmembrane helices (TM) often depends on which prediction program is used.

c The crystal structure of RibU from *Staphylococcus aureus*, ThiT and BioY from *L. lactis* show that there are six transmembrane helices (Zhang et al., 2010; Erkens et al., 2011) and chapter 4).

d Reassigned in the database, earlier named HmpT and predicted to be involved in the transport of a thiamin precursor.
Figure 3.2. Purification of EcfAA’T complexes. Coomassie Blue-stained SDS-polyacrylamide gels showing the purified fractions after nickel-Sepharose and size-exclusion chromatography. A EcfAA’T was expressed in *L. lactis* (lane 1) or in *E. coli* (lane 2). B EcfAA’T was co-produced with eight S-components in *E. coli*. BioY2 is not shown but behaved in the same way as BioY. The identities of the S-components were confirmed by Western blotting and detection using anti-STREPII tag antibodies (bottom panel). The Western blot was used only for qualitative purposes, and the amounts of protein loaded on the corresponding SDS-polyacrylamide gel were not the same as on the Coomassie Blue-stained gel.

Co-purification of the different endogenous S-components with the energizing module introduced heterogeneity and complicated structural analysis. Therefore, we decided to overproduce the energizing module in *E. coli* MC1061, a strain devoid of endogenous ECF-type ABC transporters or S-components. The Histagged energizing module EcfAA’T was again purified by nickel affinity and sizeexclusion chromatography. Analysis by SDS-PAGE (figure 3.2a) revealed that only the band corresponding to the His-tagged subunit (EcfT) was visible, indicating that the energizing module had not formed a stable complex or that the complex had fallen apart during the purification in detergent solution.

Possibly, the energizing module can form stable complexes only if an S-component is attached. To test this hypothesis, we co-produced each of the eight S-components (containing a C-terminal STREPII tag) with the EcfAA’T-His module in *E. coli* MC1061. Membrane solubilization followed by metal affinity and size-exclusion chromatography resulted in co-purification of the entire complex containing both the energizing module and the co-produced S-component (figure 3.2b).

The subunit stoichiometry of the complexes containing the EcfA, EcfA’, and EcfT and the S-components is not known (e.g. see Ref. (Finkenwirth et al., 2010)). It is possible that the S-component and EcfT together form the membrane pore (in a 1:1 stoichiometry) and associate with a heterodimer of the two ATPases (EcfA and EcfA’). In this case, the S-component would form an integral part of the complex.
On the other hand, it is also possible that two EcfT subunits form a complex with EcfA and EcfA’ (2:1:1 stoichiometry) and that the S-component is attached only peripherally, possibly in multiple copies. The latter organization could resemble sulfonylurea receptors (SURs). SURs are complete ABC transporters, with two transmembrane domains and two nucleotide-binding domains, that associate with an unrelated membrane protein (in this case with K$_{ATP}$ channels) (Bryan et al., 2007).

To determine the molecular weight of the complexes (and thus the subunit stoichiometry), it was not possible to use the elution volumes from a size-exclusion column, which had been calibrated with globular protein markers because the amount of attached detergent was not known. Instead, we determined the subunit stoichiometries of three complexes (EcfAA'T-NiaX, EcfAA'T-BioY, and EcfAA'T-ThiT) by size exclusion chromatography coupled to multi angle laser light scattering (SEC-MALLS). SEC-MALLS explicitly accounts for the amount of detergent bound to a membrane protein and allows for determination of the absolute molecular mass of a protein in a protein-lipid-detergent mixed micelle. The technique does not make use of the elution volume from the size-exclusion column. SEC is used only to separate the protein of interest from different species (contaminants/excess empty detergent micelles/aggregated proteins) (Slotboom et al., 2008; Wen et al., 1996).

The mass of the EcfAA'T-NiaX protein complex was determined throughout the elution peak and was found to be $\sim$119 kDa (shown in figure 3.3a). The only subunit stoichiometry consistent with the data was a 1:1:1:1 ratio between the ATPases EcfA and EcfA’, the transmembrane protein EcfT, and the S-component NiaX, which has a calculated mass of 120.5 kDa (table 3.2). The same quaternary structure was found for EcfAA'T-BioY and EcfAA'T-ThiT (tables 3.3 and 3.4). In all three cases, a stoichiometry in which two EcfT subunits would be present, in addition to one or more S-components, was not consistent with the data.

For EcfAA'T-BioY and EcfAA'T-ThiT, the complexes with 1:1:1:1 stoichiometry fitted best to the experimental data, although hypothetical complexes consisting of three ATPase subunits also fitted reasonably well (tables 3.3 and 3.4). However, we do not expect three ATPases to be present in the complex. The EcfA and EcfA’ are typical ABC transporter ATPases, and it is well known that these ATPases form dimers, with the active sites on the dimer interface (chapter 1). Furthermore, on the Coomassie Brilliant Blue-stained SDS-polyacrylamide gels (figure 3.2), the
Figure 3.3. Subunit stoichiometry of the EcfAA'T-S-component complex. A SEC-MALLS analysis of the EcfAA'T-NiaX complex. The chromatogram of a size-exclusion chromatography run is shown. The black trace is the signal from the differential refractive index detector. The calculated masses of protein (blue), detergent (green), and total (red) of the protein-detergent micelle are shown in the chromatogram. B Schematic representation of an ECF-type importer. The positions of EcfA and EcfA' relative to the membrane subunits are not known. S indicates S-component; the black circle indicates substrate.

Table 3.2. Subunit stoichiometry of EcfAA'T-NiaX

<table>
<thead>
<tr>
<th>EcfA</th>
<th>EcfA'</th>
<th>EcfT</th>
<th>NiaX</th>
<th>Absorbance</th>
<th>In silico calculated MW (kDa)</th>
<th>MW from SEC-MALLS (kDa)</th>
<th>Difference (kDa)</th>
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<tr>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0.683</td>
<td>120.5</td>
<td>119 +/- 3</td>
<td>-1.5</td>
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<tr>
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<td>1</td>
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<td>0.768</td>
<td>127.2</td>
<td>107 +/- 4</td>
<td>-20.2</td>
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<td>1</td>
<td>2</td>
<td>1</td>
<td>0.760</td>
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<td>1</td>
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<td>2</td>
<td>1</td>
<td>0.679</td>
<td>215.3</td>
<td>121 +/- 4</td>
<td>-94.3</td>
</tr>
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</table>

a Stoichiometric coefficient of each subunit in the EcfAA'T-NiaX complex.
Absorbance was calculated on the assumption that all cysteines were reduced.
c The MW indicated here is the weight-averaged molecular mass of the protein without the detergent contribution.

Table 3.3. Subunit stoichiometry of EcfAA'T-ThiT

<table>
<thead>
<tr>
<th>EcfA</th>
<th>EcfA'</th>
<th>EcfT</th>
<th>ThiT</th>
<th>Absorbance</th>
<th>In silico calculated MW (kDa)</th>
<th>MW from SEC-MALLS (kDa)</th>
<th>Difference (kDa)</th>
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</table>

a Stoichiometric coefficient of each subunit in the EcfAA'T-ThiT complex.
Absorbance was calculated on the assumption that all cysteines were reduced.
c The MW indicated here is the weight-averaged molecular mass of the protein without the detergent contribution.
Table 3.4. Subunit stoichiometry of EcfAAT-BioY

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</table>

a Stoichiometric coefficient of each subunit in the EcfAAT-BioY complex.
c The MW indicated here is the weight-averaged molecular mass of the protein without the detergent contribution.

bands for EcfA and EcfA’ were equally intense. The two proteins are soluble and homologous (identity is 35%) and therefore likely to stain in a similar way. It is thus likely that EcfA and EcfA’ are present in a 1:1 ratio.

The purified complexes EcfAAT-NiaX and EcfAAT-RibU were reconstituted in liposomes to investigate whether the protein complexes found in detergent solution sufficed for transport. Uptake of radiolabeled niacin (by EcfAAT-NiaX) or riboflavin (by EcfAAT-RibU) into the proteoliposomes was indeed observed. Transport depended on the presence of Mg-ATP in the lumen of the liposomes. The accumulation levels of niacin and riboflavin were 8.7- and 26-fold, respectively. Inclusion of only Mg$^{2+}$ ions or Mg-ADP in the liposome lumen did not result in substrate transport (figure 3.4). Also, the presence of 10 mM Mg-AMP-PNP, a slowly hydrolyzable ATP analogue, did not support substrate accumulation, showing that ATP hydrolysis was required (data not shown). As expected, riboflavin was not transported by the EcfAAT-NiaX complex.

In a complementary experiment, we measured transport by an efflux assay using proteoliposomes in which the orientation of the EcfAAT-NiaX complexes (right-side-out or inside-out) was deliberately scrambled (Geertsma et al., 2008). Importantly, upon the addition of external Mg-ATP, luminal niacin was rapidly released from the liposomes (figure 3.5), confirming that the reconstituted complexes mediated substrate translocation in the presence of Mg-ATP.
Figure 3.4. Transport of [$^3$H]niacin (A) and [$^3$H]riboflavin (B) into proteoliposomes containing EcfAA'T-NiaX and EcfAA'T-RibU, respectively. Error bars indicate the standard error of three measurements. The proteoliposomes were loaded with 50 mM potassium phosphate, supplemented with 10 mM MgSO$_4$ and 10 mM ATP (closed circles), 10 mM MgSO$_4$ only (open circles), or 10 mM MgSO$_4$ and 10 mM ADP (closed triangles). The pH was 7. The accumulation level of niacin (at the 3 min timepoint) and riboflavin (at the 6 minutes timepoint) are 8.7- and 26-fold, respectively. The non-zero levels of radioactivity (riboflavin or niacin) counted using the proteoliposomes loaded with Mg-ADP or MgSO$_4$ only are due to non-specific binding of the label to the proteoliposomes. Similar levels were observed when proteoliposomes of an unrelated protein were used (the secondary active aspartate transporter GltPh, data not shown).

Figure 3.5. Efflux of niacin from EcfAA'T-NiaX proteoliposomes. For the efflux experiments proteoliposomes containing EcfAA'T-NiaX with a scrambled orientation were used (Geertsma et al., 2008). The proteoliposomes were incubated for four hours at 6°C with [$^3$H]niacin to equilibrate the radiolabel between the medium and vesicle lumen. Then the suspension was diluted in buffer and incubated at 25°C. Aliquots were taken at indicated time points to determine the amount of [$^3$H]niacin that was left in the lumen (half black/half white circles). After 3 min the sample was split and either 9.1 mM Mg-ATP was added (black circles) or, as a control, buffer was added (open circles).
3.5 Discussion

For the first time, we have shown in a comprehensive genome-wide analysis that all predicted S-components in a single organism (BioY, BioY2, NiaX, PanT, PdxU2, QueT, RibU, and ThiT from *L. lactis*) indeed interact with the same energizing module. With the exception of BioY and BioY2, the S-components do not share significant sequence similarity, raising the question how these proteins recognize the same EcfAA'T module. Recent crystal structures of RibU (Zhang et al., 2010), ThiT (Erkens et al., 2011) and BioY (chapter 4), which showed that the S-components have a similar fold, have provided the first tentative answer to this question. All three structures show a tight bundle of six transmembrane helices. No coupling helix could be found in any of the S-component structures, and therefore we hypothesized that binding of the S-component with the EcfAA'T-complex may be mediated mostly by interaction between the S-component and EcfT. Helices 1-3 are more structurally conserved between the different S-components then helices 4-6 that form the specific binding pocket for the substrate. Therefore, helices 1-3 are expected to interact with the EcfT. Helix 1 contains an AxxxA motif that was shown to be essential for thiamin transport in the EcfAA'T-ThiT complex but not for thiamin binding. From the work presented in this chapter, it is clear that the interactions between the energizing module and the S-components are strong and that the EcfAA'T module is stable in detergent only when an S-component is attached.

The observed subunit stoichiometry of the purified complexes (1:1:1:1 EcfA:EcfA′: EcfT:S-component) shows that the S-component is an integral part of the ECF transporter taking the place of one of the two transmembrane domains found in classical ABC transporters (figure 3.3b), which may explain why EcfAA'T alone did not form a stable complex in the absence of an S-component (figure 3.2a). The quaternary structure makes ECF transporters clearly different from the SURs, to which the $K_{ATP}$ channels are attached peripherally (Bryan et al., 2007). The presence of a single S-component in the complex is consistent with results showing that the S-components ThiT and BioY are monomeric in detergent solution in the absence of the energizing module and that monomeric ThiT and BioY bind their substrate in a 1:1 ratio (Erkens & Slotboom, 2010) (chapter 4). It also agrees with the recent crystal structures of RibU, ThiT and BioY that have a monomeric functional unit (Zhang et al., 2010; Erkens et al., 2011) (chapter 4). The results are not directly compatible with FRET lifetime measurements in *E. coli* cells expressing...
the biotin transporter BioMNY (which has a dedicated energizing module). A multimeric state was found for the S-component BioY, both alone and in complex with its dedicated energizing module (Finkenwirth et al., 2010). It is possible that the structure of BioMNY is different from the ECF-type transporters with shared energizing modules studied here. On the other hand, it is also possible that the basic unit with a 1:1:1:1 subunit stoichiometry forms higher order arrays as part of the transport cycle. Our SEC-MALLS analysis indicated that the complexes have a tendency to form higher order aggregates in detergent solution. Besides the main protein peak in the chromatogram (at 12.3 ml), a shoulder (at 11.1 ml) was also present that eluted earlier. The protein complex in this peak had a calculated molecular mass of exactly twice the mass of the protein in the main peak (figure 3.3a). Likely, the protein peak eluting at 11.1 ml represents a dimer of two EcfaAT-NiaX complexes, and the data indicate that the complex has a tendency to aggregate in detergent solution (see also chapter 6).

The uptake and efflux experiments revealed that the reconstituted complexes with a 1:1:1:1 quaternary structure (Ecfa:EcfA':Ecft:S-component) mediated ATP dependent transport without the need for any soluble substrate-binding domains, which are employed by other types of ABC import proteins. Thus, the ECF-type ABC transporters described here represent the smallest functional unit of any ABC importer found so far, with a total molecular mass of around 120 kDa. The use of eight different S-components and a single energizing module further adds to the minimalist properties of these transporters.