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.

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

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EXPERIMENTAL PROCEDURES

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), here renamed as ecfA, ecfA', and ecfT) was cloned in a pNZ28048 vector (9) for expression in the L. lactis strain NZ9000 (10) and in a pBAD vector (11, 12) for expression in the Escherichia coli strain MC1061 (13). The sequence coding for a His10 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 (12). For simultaneous expression of the ecf operon and the genes encoding S-components in E. coli MC1061, an expression vector based

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2 The abbreviations used are: ABC, ATP-binding cassette; ECF, energy coupling factor; SEC-MALLS, size-exclusion chromatography coupled to multi-angle laser light scattering; SUR, sulfonylurea receptor; AMP-PNP, adenosine 5'-(β,y-imino)triphosphate.
on pBAD24 was used with two tandem arabinose-inducible promoters, ribosomal-binding sites, multiple cloning sites, and terminator sequences.3 Downstream of the first promoter, the ecfAA’T- His operon was cloned. Downstream of the second promoter, a gene coding an S-component was cloned in-frame with the sequence coding for a C-terminal STREPII tag (WSHPQFEK). Cultivation, induction of expression, and harvesting of E. coli and L. lactis cells as well as preparation of membrane vesicles were done according to standard protocols (see supplemental methods).

**Protein Purification**—The His-tagged complexes EcfaA’T and EcfaA’T-S-component were solubilized in 0.5% n-dodecyl-β-d-maltoside and purified in two steps, using nickel-Sepharose and size-exclusion chromatography. Details of the protein purification procedure can be found in the supplemental methods.

**Light Scattering**—The subunit stoichiometries of the complexes were determined by size-exclusion chromatography coupled to multi-angle laser light scattering (SEC-MALLS) (14, 15). SEC-MALLS was performed as described before (6, 14). 200 µl of the purified protein was used in the experiment (~150 µg). 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. (15). 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.

**Analysis by SDS-PAGE, Western Blotting, and MS/MS**—SDS-PAGE, Western blotting, and protein immunodetection were done according to standard protocols (for detailed information, see supplemental methods). 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. (16). The peptide digests were mixed 1:1 (v/v) with a solution of α-cyano-4-hydroxycinnamic acid matrix (5 mg/ml in 50% acetonitrile and 0.1% TFA, LaserBio Labs), spotted onto a stainless steel MALDI target, and analyzed with a 2800 TR isotope counter.

**Reconstitution and Transport Assays in Proteoliposomes**—The purified complexes EcfaA’T-NiaX and EcfaA’T-RibU were reconstituted into proteoliposomes, using the method described by Geertsma et al. (17). 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 (EcfaA’T-NiaX for uptake experiments), 1:100 (EcfaA’T-NiaX for efflux experiments), and 1:333 (EcfaA’T-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 MgSO4, 10 mM ADP and 10 mM MgSO4, 10 mM MgSO4 only, or 10 mM AMP-PNP with 10 mM MgSO4. 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 × g, 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 EcfaA’T-NiaX, [3H]niacin was added to a final concentration of 375 nM, and in the case of EcfaA’T-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.

**RESULTS**

The genome of the Gram-positive bacterium L. lactis contains one operon coding for a shared energizing module (ecfaA’T, annotated as cbiOOQ2). In addition, it contains eight different genes coding for predicted S-components with confirmed or predicted specificity for vitamins and their precursors (5–7) (Table 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 (18). His-tagged EcfaA’T was produced in L. lactis, solubilized, and purified by nickel affinity and size-exclusion chromatography. Fig. 1a 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

### Table 1

| S-component found in L. lactis and predicted to interact with the shared energizing module (5) | a.a., amino acids |
|-----------------------------------------------|------------------|
| Name (Probable) substrate | Size | Predicted number of TM helices | Accession code in genome |
| BioY Biotin | 189 | 20.5 | 6 | llmg_1964 |
| BioY2 Biotin | 182 | 19.7 | 6 | llmg_0332 |
| HmpT Thiamine precursor | 166 | 18.1 | 4 or 5 | llmg_0464 |
| NiaX Niacin | 222 | 24.6 | 5 or 6 | llmg_1130 |
| PanT Pantethenic acid | 196 | 21.1 | 6 | llmg_0542 |
| QueT Queosine precursor | 169 | 19.1 | 4 or 5 | llmg_1760 |
| RibU Riboflavin | 206 | 23.0 | 5 or 6 | llmg_1195 |
| ThfT Thiamine | 182 | 19.9 | 5 or 6 | llmg_0334 |

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**Quaternary Structure of ECF Transporters**

The crystal structure of RibU from *Staphylococcus aureus* shows that there are six transmembrane helices (8).
had formed stable complexes with the energizing modules. Indeed, one of the proteins (indicated in Fig. 1a) 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.

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 His-tagged energizing module EcfAA’T was again purified by nickel affinity and size-exclusion chromatography. Analysis by SDS-PAGE (Fig. 1a) revealed that the only 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 (Fig. 1b).

The subunit stoichiometry of the complexes containing the EcfA, EcfA’, and EcfT and the S-components is not known (e.g. see Ref. 19). 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 $K_{\text{ATP}}$ channels) (20).

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 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) (14, 15). The mass of protein complex was determined throughout the elution peak and was found to be $\sim 119$ kDa (shown in Fig. 2a for EcfAA’T-NiaX). 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 2). The same quaternary structure was found for EcfAA’T-BioY and EcfAA’T-ThiT (supplemental Tables 1 and 2). 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 (supplemental Tables 1 and 2). 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. Furthermore, on the Coomassie Brilliant Blue-stained SDS-polyacrylamide gels (Fig. 1), the 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.

**FIGURE 1.** 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 seven 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.
The purified complexes Ecfa\textsubscript{A}’T-NiaX and Ecfa\textsubscript{A}’T-RibU were reconstituted in liposomes to investigate whether the protein complexes found in detergent solution sufficed for transport. Uptake of radiolabeled niacin (by Ecfa\textsubscript{A}’T-NiaX) or riboflavin (by Ecfa\textsubscript{A}’T-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\textsuperscript{2+} ions or Mg-ADP in the liposome lumen did not result in substrate transport (Fig. 3). 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 Ecfa\textsubscript{A}’T-NiaX complex.

In a complementary experiment, we measured transport by an efflux assay using proteoliposomes in which the orientation of the Ecfa\textsubscript{A}’T-NiaX complexes (right-side-out or inside-out) was deliberately scrambled (17). Importantly, upon the addition of external Mg-ATP, luminal niacin was rapidly released from the liposomes (supplemental Fig. 1), confirming that the reconstituted complexes mediated substrate translocation in the presence of Mg-ATP.

**TABLE 2**

| Ecfa\textsuperscript{A} | Ecfa\textsuperscript{A}’ | Ecft\textsuperscript{a} | NiaX\textsuperscript{a} | Extinction coefficient\textsuperscript{b} | In silico calculated M\textsubscript{w}\textsuperscript{b} | M\textsubscript{w} from SEC-MALLS\textsuperscript{c} | Difference |
|-------------------------|-------------------------|-------------------------|-------------------------|---------------------------------|--------------------------|--------------------------|----------|
| 1 1 1 1 1 0.683 120.5 119 ± 3 | 1.5 | 1 1 2 0 0.768 127.2 107 ± 4 | 20.2 | 1 1 2 2 1 0.689 146.2 118 ± 3 | 28.2 | 1 1 2 1 1 0.636 152.1 129 ± 4 | 23.1 |
| 2 2 2 1 0.679 215.3 121 ± 4 | 94.3 | 1 2 1 1 0.636 215.3 121 ± 4 | 94.3 | 2 1 1 1 | 0.646 151.4 127 ± 4 | 24.8 | 1 2 1 1 0.636 152.1 129 ± 4 | 23.1 |

\textsuperscript{a} Stoichiometric coefficient of each subunit in the Ecfa\textsubscript{A}’T-NiaX complex.

\textsuperscript{b} Calculated via the ProtParam tool on the ExPASy Proteomics Server. Extinction coefficients were calculated on the assumption that all cysteines were reduced.

\textsuperscript{c} M\textsubscript{w} is the weight-averaged molecular mass of the protein without the detergent contribution. The values are the averages of two independent experiments and the error indicates the range.

**FIGURE 2.** Subunit stoichiometry of the Ecfa\textsubscript{A}’T-S-component complex. \(a\), SEC-MALLS analysis of the Ecfa\textsubscript{A}’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.

**FIGURE 3.** Transport of \([^{3}H]\)niacin (\(a\)) and \([^{3}H]\)riboflavin (\(b\)) into proteoliposomes containing Ecfa\textsubscript{A}’T-NiaX and Ecfa\textsubscript{A}’T-RibU, respectively. Error bars indicate the S.E. of three measurements. The proteoliposomes were loaded with 50 mM potassium phosphate, supplemented with 10 mM MgSO\textsubscript{4} and 10 mM ATP (closed circles), 10 mM MgSO\textsubscript{4} only (open circles), or 10 mM MgSO\textsubscript{4} and 10 mM ADP (closed triangles). The pH was 7. The accumulation levels of niacin (at the 3-min time point) and riboflavin (at the 6-min time point) 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\textsubscript{4} only are due to nonspecific binding of the label to the proteoliposomes. Similar levels were observed when proteoliposomes of an unrelated protein were used (the secondary active aspartate transporter Glt\textsubscript{Ph}, data not shown).
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, HmpT, NiaX, PanT, 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 EcFA`T module. Presumably, there is a structurally conserved motif in the S-components that is responsible for the interaction with either the EcTT protein or a nucleotide-binding domain or both. It is not clear from the amino acid sequences whether the S-components contain a motif related to the coupling helix that mediates binding between the membrane domains and ATPase subunits in classical ABC transporters. The crystal structure of RibU from S. aureus also did not reveal a coupling helix or any obvious binding sites for the S-component (8). Nonetheless, from our work, it is clear that the interactions between the energizing module and the S-components are strong and that the EcFA`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`EcTT: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 (Fig. 2b), which may explain why EcFA`T alone did not form a stable complex in the absence of an S-component (Fig. 1a). The quaternary structure makes ECF transporters clearly different from the SURs, to which representatives of two EcFA`T-NiaX complexes, and the data indicate that the complex has a tendency to aggregate in detergent solution.

The uptake and efflux experiments revealed that the reconstituted complexes with a 1:1:1:1 quaternary structure (EcFA`EcTT:S-component) mediated ATP-dependent transport without the need for any soluble substrate-binding domains, which are employed by classical 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 minimalistic properties of these transporters.

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