The ABC of ECF transporters

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Chapter 6

Biochemical characterization of EcfAA’T-S-component complexes from \textit{L. lactis}

6.1 Abstract

EcfAA’T-S-component complexes were investigated \textit{in vivo} and \textit{in vitro}. \textit{E. coli} cells expressing the shared ECF module (EcfAA’T) from \textit{L. lactis} together with the S-components NiaX or ThiT transport niacin and thiamin, respectively. Here, we show that the same ECF module did not catalyze transport of biotin when co-expressed with one of the two related S-components BioY or BioY2. Instead, only biotin binding to BioY or BioY2 was detected. Also for EcfAA’T-RibU and EcfAA’T-PanT transport of riboflavin and pantothenic acid, respectively, could not be measured in \textit{E. coli}. Possibly, heterologous expression in \textit{E. coli} does not exactly mimic the conditions in \textit{L. lactis}, e.g. because interactions between the EcfAA’T and the S-component complex may be dependent on lipid composition.

When solitary S-components: RibU (S-component for riboflavin) (Duurkens et al., 2007), ThiT (S-component for thiamin) (Erkens & Slotboom, 2010; Erkens et al., 2011) and BioY (S-component for biotin, chapter 4) were purified from \textit{L. lactis} cultivated on a medium that contained their substrates, only substrate-bound proteins were obtained. In contrast, the purified EcfAA’T-RibU, EcfAA’T-ThiT
and EcfAA’T-BioY complexes were substrate-free even though the proteins were expressed in cells growing in a medium that contained riboflavin, thiamin and biotin. This observation may indicate that substrates bind with lower affinity when the S-components are in a complex with the ECF module.

When EcfAA’T-ThiT in detergent solution was supplemented with thiamin, the complexes aggregated. Similar results were obtained when niacin and riboflavin were added to EcfAA’T-NiaX and EcfAA’T-RibU, respectively. The results may indicate that the EcfAA’T-S complexes form higher order oligomers during the translocation cycle, but another possibility is that the substrate-bound complexes adopt a conformation that is not stable in detergent solution. Dissociation of the S-component from the ECF module in detergent solution could not be observed in any of the conditions tested (with/without nucleotides and/or substrates).

The aggregation of the complexes is a problem for the biochemical characterization of the EcfAA’T complexes in vitro. Substrate binding measurements to EcfAA’T-BioY or EcfAA’T-RibU complexes, using changes in intrinsic protein fluorescence or riboflavin fluorescence in case of RibU, were unsuccessful because the signals were not stable over time. We suspect that these problems are due to protein aggregation. Nonetheless, preliminary results indicate that the addition of MgATP increases the number of binding sites and/or the substrate binding affinity. An EcfAA’T-ThiT double mutant, with the glutamates at the end of the Walker B motifs of both ATPase domains changed into glutamines (EcfA:E166Q and EcfA’:E170Q), did not show ATPase activity. Mutations in single ATPase subunits maintained around 40% activity in the EcfAA’T-ThiT complex. For further biochemical characterization it will be necessary to find conditions that stabilize the EcfAA’T-S-components.

6.2 Introduction

ABC transporters are membrane proteins that use the hydrolysis of ATP to transport substrate across the membrane. The distinguishing characteristic of ABC transporters is the presence of two nucleotide-binding domains (NBDs or ATPases) that bind and hydrolyze ATP. In addition to the NBDs, ABC transporters always contain two transmembrane domains (TMDs). ABC transporters may be exporters or importers. In exporters substrates are transported to the trans side of the membrane (relative to the NBDs, for bacteria this is to the outside of the cell), whereas importers translocate their substrates in the opposite direction (towards
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the side of the NBDs, for bacteria this is to the inside of the cell). Exporters are found in all organisms, while importers are found only in prokaryotes. Based on structural analysis, one group of exporters has been identified, while there are three groups of ABC importers (chapter 1). Type I and II ABC importers depend on an additional substrate-binding domain or protein (SBP). This SBP is a soluble domain or protein, diffusing in the periplasm (Gram-negative bacteria), or located at the outside of the cell and linked to the membrane via a lipid anchor or direct connection to the TMDs of the transporter (Gram-positives and Archaea). Type III or ECF-type ABC importers do not employ an extracytoplasmic SBP, but bind their substrate in the membrane.

In ECF-type importers one of the TMDs, called the S-component, contains a high-affinity substrate-binding site. The rest of the transporter, called the ECF module, contains two ATPases (identical or homologous, EcfA and EcfA') and a second TMD (EcfT). For dedicated ECF modules the genes coding for the ECF module are organized in an operon that also contains the gene coding for a single S-component (chapter 2). The encoded proteins have been shown to form active ECF-S-component transporting complexes (Hebbeln et al., 2007). The genes coding for shared ECF modules are organized in an operon without any genes for S-components (chapter 2). Shared ECF modules (consisting of EcfAA’T or EcfA2’T proteins) have been shown to interact with multiple S-components, encoded by genes that are scattered around the genome (chapter 2 and 3). Depending on which S-component is bound by the ECF module, the EcfAA’T-S complexes transport particular substrates in an ATP-dependent manner (chapter 3 and 4).

Previously, we have shown that EcfAA’T-S-component complexes from \textit{L. lactis} can be expressed in \textit{E. coli} and purified in detergent solution (chapter 3). The subunit stoichiometry of these complexes was found to be 1:1:1:1 (EcfA:EcfA’:EcfT:S-component). The complexes could be reconstituted in an active state into proteoliposomes. Results presented in chapter 5 indicate that the EcfAA’T-NiaX (S-component for niacin) and EcfAA’T-ThiT (S-component for thiamin) complexes are dynamic: ThiT bound to the ECF module can be exchanged for NiaX and both S-components bind stronger to the EcfAA’T when their substrate is present. Here, we further characterized EcfAA’T-S-component complexes from \textit{L. lactis}. We studied substrate transport by the complexes expressed in \textit{E. coli}, and substrate binding to the complexes \textit{in vitro}, using ECF complexes with BioY or BioY2 (both S-components for biotin), RibU (S-component for riboflavin), ThiT (S-component for thiamin). In addition, we tested whether or not isolated EcfAA’T-ThiT complexes could be dissociated by the addition of substrate and/or nucleotides.
6.3 Materials & Methods

6.3.1 Protein expression and transport assays.

EcfAA'T-S-component complexes were expressed in *E. coli* as described previously (chapter 3). 2 hours after induction of expression with $10^{-3}\%$ (w/v) L-arabinose at $25^\circ C$, the cells were spun down and transport assays were done as described before (chapter 5). For biotin uptakes $21 \text{nM} [^3\text{H}]\text{biotin}$ (American Radiolabeled Chemicals, ARC) was added at $t = 0$ and after 19 min $0.9 \text{mM}$ unlabeled biotin was added. For riboflavin uptake $33 \text{nM} [^3\text{H}]\text{riboflavin}$ (ARC) was added at $t = 0$ min and $9.5 \text{µM}$ riboflavin at $t = 30$ min. For pantothenic acid uptake $17 \text{nM} [^3\text{H}]\text{pantothenic acid}$ (ARC) was added at $t = 0$ min and $1 \text{mM}$ pantothenic acid at $t = 30$ min.

6.3.2 Expression media.

M17 (Difco) contains $0.5\%$ (w/v) pancreatic digest of casein, $0.5 \%$ (w/v) soy peptone, $0.5 \%$ (w/v) beef extract, $0.25 \%$ (w/v) yeast extract, $0.05 \%$ (w/v) ascorbic acid, $0.025 \%$ (w/v) MgSO$_4$ plus $1.9 \%$ (w/v) disodium-glycerophosphate and was supplemented with $0.5-1 \%$ (w/v) glucose. GLS contains $2 \%$ (w/v) yeast extract (gistex LS, Strik BV, Eemnes, NL), $65 \text{mM}$ sodium phosphate, pH 6.5 plus $1 \%$ (w/v) glucose. Luria Broth (LB) contains $1 \%$ (w/v) bacto$^\text{TM}$tryptone, $0.5 \%$ (w/v) BBL$^\text{TM}$ yeast extract and $1 \%$ NaCl. 2xTY medium contains $1.6 \%$ (w/v) bacto$^\text{TM}$tryptone, $1 \%$ (w/v) BBL$^\text{TM}$ yeast extract and $0.5 \%$ NaCl and was supplemented at high optical densities with $0.2 \%$ glycerol to support further growth. All these media thus contain yeast extract, which is the water soluble portion of autolyzed yeast and has been indicated as a source of vitamin B complex. This means that all eight B vitamins are present: including thiamin (vitamin B$_1$), riboflavin (vitamin B$_2$) and biotin (vitamin B$_7$).

6.3.3 Protein purification.

For *in vitro* characterization EcfAA'T-S-component complexes were overexpressed in and purified from *E. coli* as described previously (chapter 3). For the ATPase assays, where phosphate could not be present, the potassium phosphate was replaced by an ammonium acetate buffer (both at pH 7.5) during the wash-step on Nickel-Sepharose and this buffer was subsequently used in all steps.
6.3.4 Biotin assay.

To analyze whether biotin was bound to BioY or EcfAA'T-BioY the FluoReporter Biotin Quantitation Assay Kit for biotinylated proteins (molecular probes) was used. The peak fractions from a gel filtration chromatography run of two independent protein purifications of EcfAA'T-BioY complexes or BioY alone were used. The concentration of EcfAA'T-BioY complexes in the assay was \( \sim 700 \text{ nM} \) (undiluted peak fraction), and for BioY three different concentrations were used: the undiluted peak fraction (2600 nM) as well as two dilutions in gel filtration buffer of 260 nM and 26 nM. 1 \( \mu \text{L} \) protease stock solution from the FluoReporter kit was added and the proteins were digested for 2 hours at 37 °C. After this step the samples were boiled for 30 minutes. Biotin standards were prepared of 1.6, 0.8, 0.4, 0.2, 0.1 and 0 \( \mu \text{M} \) (by diluting the 200 \( \mu \text{M} \) biocytin stock from the kit in phosphate buffered saline, PBS). 50 \( \mu \text{L} \) of the standards and the samples (in duplicates) were pipetted into a black Costar 96-well plate with a glass bottom (Corning). Then the reaction was started by adding 50 \( \mu \text{L} \) reconstituted 2x Biotective Green reagent to each of the filled wells. The plate was shaken carefully, wrapped in aluminum foil to protect it from light and incubated for 5 minutes at room temperature. The fluorescence of the wells was then measured with a Cary Eclipse Fluorescence Spectrophotometer (Varian Inc.) using an excitation wavelength of 485 nm and an emission wavelength of 530 nm.

6.3.5 Fluorescence titration.

Tryptophan fluorescence was measured in a 1000 \( \mu \text{L} \) stirred quartz cuvette on a Spex Fluorlog 322 fluorescence spectrophotometer (Jobin Yvon) at 25 °C. The excitation wavelength was 280 nm for tryptophan fluorescence and the emission wavelength is indicated in the figure legends. For riboflavin fluorescence the excitation wavelength was 435 nm and the emission wavelength was 523 nm. Protein was diluted to the indicated concentration in gel filtration buffer and incubated for 5 min to reach temperature equilibrium. The substrates were added in 0.5-2.0 \( \mu \text{L} \) steps by hand or using a syringe pump (Harvard apparatus) fitted with a 500 \( \mu \text{L} \) gastight glass syringe (Hamilton Co.). The syringe was connected to the cuvette by tubing with an internal diameter of 0.13 mm (Vici AG International). For the riboflavin fluorescence measurements the additions were done by hand. The fluorescence signals were averaged over a period of 20 s. After each substrate
addition, the first 5 s interval was not taken into account to avoid mixing effects.

The change in tryptophan fluorescence was calculated and corrected for the dilution. This was done via equation 6.1:

$$\Delta F_i = |F_0 \left( \frac{V_0}{V_0 + d_i} \right) - F_i|$$  \hspace{1cm} (6.1)

$\Delta F_i$ = The change in tryptophan fluorescence at step $i$ corrected for dilution (A.U.)
$V_0$ = Initial sample volume ($\mu$L)
$d_i$ = The total volume that was added during the titration, at step $i$ ($\mu$L)
$F_0$ = Average fluorescence before the first addition (A.U.)
$F_i$ = Average fluorescence after the $i$th titration (A.U.)

The $\Delta F_i$ was then plotted against the substrate concentration and fitted with equation 6.2 in Origin 7.0 (OriginLab).

$$\Delta F_i = A \left( \frac{n + [S] + K_d}{2} - \frac{\sqrt{(n + [S] + K_d)^2 - 4n[S]}}{2} \right)$$  \hspace{1cm} (6.2)

$\Delta F_i$ = The change in tryptophan fluorescence corrected for dilution (A.U.)
$A$ = Proportionality factor
$n$ = Concentration of binding sites in the cuvette (M)
$[S]$ = Substrate concentration in the cuvette (M)
$K_d$ = Dissociation constant (M)

To correct for the dilution of substrate in the cuvette equation 6.3 was inserted into the above formula and for correction of the protein equation 6.4 was used.

$$[S] = [S]’ \left( \frac{V_0}{V_0 + d_i} \right)$$  \hspace{1cm} (6.3)

$[S]'$ = The substrate concentration in the cuvette not corrected for dilution (M)

$$[n] = [n_0] \left( \frac{V_0}{V_0 + d_i} \right)$$  \hspace{1cm} (6.4)

$[n_0]$ = The concentration of binding sites at the start of the titration
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For medium to low affinity ($K_d > 10 \times $ protein concentration) binding equation 6.5 was used instead of equation 6.2.

$$\Delta F = \frac{B[S]}{K_d + [S]}$$

(6.5)

$B$ = maximal change in fluorescence (A.U.) at saturating substrate concentrations

$[S]$ = substrate concentration in the cuvette corrected for dilution via equation 6.3

For riboflavin fluorescence the change in fluorescence ($\Delta F$) of the substrate rather than protein was measured. The change of riboflavin fluorescence in a protein solution was subtracted from the $\Delta F$ of riboflavin in a buffer solution. The substrate concentration was then corrected for the dilution via equation (6.3) and then $\Delta F$ was plotted against [S] and fitted with equation 6.2 in Origin 7.0 (OriginLab).

### 6.3.6 ATPase assays.

ATPase activity was measured via the malachite green phosphate assay kit (POMG-25H, BioAssay Systems) and the protocol for a 96 well plate was followed. Absorbance was measured at 620 nm in a plate reader (BioTek PowerWave 340).

### 6.4 Results

#### 6.4.1 Transport assays in *E. coli* cells

In the previous chapter uptake of thiamin and niacin by *E. coli* cells overexpressing ThiT or NiaX, respectively, were shown in the presence or absence of the ECF module. The substrates were transported into the cells, only when the EcfAA'T was coexpressed with the S-components. Here we performed similar analyses for other S-components of which the substrates are known: BioY (biotin), BioY2 (biotin), RibU (riboflavin) and PanT (pantothenic acid). This data is shown in figures 6.1 and 6.2.

For biotin uptake by BioY (figure 6.1a) there is no difference between the EcfAA'T-BioY expressing cells and the cells expressing BioY only. In both cases only biotin binding to the cells seems to take place, since radioactivity is rapidly decreased to
background levels when non-labeled biotin is added in excess. Most biotin was bound by cells expressing BioY, ($\sim$1.5 pmol biotin per OD unit per mL). Assuming that there are 109 *E. coli* cells per OD unit per mL and deducting the background of 0.2 pmol biotin OD$^{-1}$ mL$^{-1}$, we estimated that $\sim$800 biotin molecules were bound per cell. The amount of membrane proteins in the inner membrane of *E. coli* has been estimated to be around 500,000 (Lindén et al., 2012). This would mean that if biotin is bound 1:1 to BioY, BioY would be over-expressed to $\sim$0.16 % of the total membrane proteins.

For biotin uptake via BioY2 there is a small difference between cells expressing BioY2 only or EcfAA'T-BioY2 (figure 6.1b). The EcfAA'T-BioY2 expressing cells show slightly higher levels of radioactivity and may release the biotin more slowly after the addition of unlabeled biotin. Nonetheless, also when EcfAA'T-BioY2 complexes were expressed, radiolabeled biotin did not get trapped and the level of radioactivity decreased to background levels upon addition of unlabeled biotin. Therefore, it seems that biotin is not really taken up but merely bound to the cells. Based on the assumptions stated above, there are $\sim$300 (for BioY2) biotin molecules bound per cell, indicating that $\sim$0.06 % of the membrane proteome is BioY2. For EcfAA'T-BioY2 expressing cells $\sim$420 biotin molecules are bound/transported.
Figure 6.2. Riboflavin (A) or pantothenic acid (B) uptake in *E. coli* cells overexpressing RibU (A) or PanT (B) alone (white circles), with the wildtype EcfAA'T-complex (black inverted triangles) or with the mutated EcfAA'T-complex, where both ATPases are inactivated (white triangles only shown in A). Black circles show the background level for the uptake levels in *E. coli* cells with an empty vector. $[^3]H$-labeled substrate was added at *t*=0 and unlabeled substrate was added at *t*=30 min.

The riboflavin levels were the same for RibU or EcfAA'T-RibU expressing cells (figure 6.2a). However, after the addition of unlabeled riboflavin there was a difference between RibU expressing cells and cells expressing EcfAA'T-RibU: the former more rapidly released radiolabeled riboflavin (indicating that it was merely bound to the outside of the cells), while EcfAA'T-RibU expressing cells retained some of the radiolabel (although also here the level of radioactivity decreased). Cells co-expressing RibU with inactivated EcfAA'T (with mutated ATPases, see below) behaved as cells expressing RibU only. Based on the assumptions stated above, there were 1800-2000 riboflavin molecules bound per cell, which (in case of 1:1 binding to RibU) indicates that $\sim0.38 \%$ of the membrane proteome is RibU. In RibU expressing cells 100 to 200 molecules of labeled riboflavin remained bound after the addition of unlabeled riboflavin, while for EcfAA'T-RibU expressing cells 700 to 1100 molecules remained associated per cell. This result might indicate that there was riboflavin transport into EcfAA'T-RibU expressing cells.

For pantothenic acid uptake there was no difference between cells expressing PanT or EcfAA'T-PanT. Both showed initial levels of radioactivity above background and a fast decline to levels below background when unlabeled pantothenic acid was
added. The background level for pantothenic acid is very high in *E. coli* cells, probably because of substrate transport and/or binding to transporters of *E. coli*. *E. coli* has a high affinity transporter that is dependent on the membrane potential (Vallari & Rock, 1985). Unlabeled pantothenic acid was not added to the control cells. The reduction of radioactivity to levels below the background upon the addition of unlabeled pantothenic acid to PanT expressing cells indicates that there may also be binding to the endogenous pantothenic acid transporter from *E. coli* or that the transport direction of this transporter is reversed upon the addition of unlabeled substrate. Using the parameters stated above we estimated that \(~900\) molecules of pantothenic acid are bound per cell because of PanT expression. This would indicate an over-expression of PanT to 0.18 % of the total membrane proteome.

Since we could not measure substrate transport by the four S-components and EcfAAT-S-component complexes, further characterization of the transport mechanism in intact *E. coli* cells was impossible. Therefore we focused on the biochemical characterization of detergent-solubilized and purified complexes.

### 6.4.2 Substrate binding

When RibU without the ECF module was expressed in *L. lactis*, grown in rich growth medium (GLS or M17), and purified, riboflavin remained bound to the protein throughout the purification procedure, even though the substrate was not added during solubilization and purification (Duurkens et al., 2007). The presence of riboflavin associated with purified RibU can be detected spectroscopically, since riboflavin has two characteristic absorption peaks at 444 nm and 372 nm (shifted to 464 and 360 nm respectively when bound to RibU) (Duurkens et al., 2007). The fact that riboflavin remained bound during the purification procedure is consistent with the subnanomolar dissociation constant \((K_d = 0.6 \text{nM})\) and indicates that the \(k_{off}\) \((s^{-1})\) of RibU for riboflavin is very small. The observation that riboflavin could not be dialyzed away readily supports this assumption (Duurkens et al., 2007).

We determined whether EcfAAT-RibU complexes isolated from cells grown on 2xTY and purified by nickel-Sepharose and size exclusion chromatography (SEC), also had riboflavin associated. 2xTY is a medium with higher amounts of yeast extract and bactotryptone than Luria Broth (LB). LB is known to contain \(~0.5 \mu\text{M}\) riboflavin (Hemberger et al., 2011) and therefore 2xTY must contain even higher
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Figure 6.3. Absorption spectrum of EcfAA’T-RibU complexes at the beginning of the elution peak from size-exclusion chromatography (black line) and at the end of the elution peak (grey line). There is no absorption at the wavelengths characteristic for riboflavin (maxima at 444 nm and 372 nm, when free in solution or 464 nm and 360 nm when bound to RibU). There is an absorption peak at 408 nm for the early peak fraction, but this may originate from a contamination that eluted faster from the column.

amounts of riboflavin. An absorbance spectrum of purified EcfAA’T-RibU (figure 6.3) did not show the characteristic peaks for riboflavin that were observed in case of RibU, but in an early fraction from size exclusion chromatography there was a small absorbance peak at 408 nm (figure 6.3). During the size exclusion chromatography run the absorbance at 280 nm and at 370 nm was measured in-line (408 nm was not measured). We found that a protein or compound absorbing at 370 nm eluted just before the EcfAA’T-RibU peak. Possibly this contaminant is also present in the elution fraction containing EcfAA’T-RibU and causes the absorption at 408 nm in figure 6.3.

To investigate whether purified EcfAA’T-BioY complexes also were devoid of substrate, isolated protein complexes from *E. coli* grown on 2xTY medium, which contains biotin, were analyzed with the FluoReporter Biotin Quantitation Assay Kit for biotinylated proteins (molecular probes). As a positive control BioY purified from *L. lactis* grown on M17, which was known to contain biotin (chapter 4) was
Figure 6.4. The amount of biotin in the BioY and EcfAA’T-BioY purification fractions reported by a FluoReporter that gives an increased fluorescence signal when biotin is present. The grey bars show samples with a known biotin concentration. As expected there is an increase of the fluorescence with the increase of the biotin concentration. The striped bars indicate the signals for purified BioY at three different protein concentrations (expressed in M17 medium). The black bars are of purified EcfAA’T-BioY complexes that were expressed in cells cultivated in 2xTY medium. Both purifications were done via Nickel-Sepharose and size-exclusion chromatography. Error bars indicate the range of two datapoints.

taken along. The sample of purified BioY indeed was found to contain biotin (the ratio between BioY and biotin was ~1:1). When six samples of the peak fraction containing 0.7 µM EcfAA’T-BioY were measured, the fluorescent signals were all around the level of the background and below the level of fluorescence that was measured for 0.1 µM biotin (figure 6.4). Therefore it was concluded that EcfAA’T-BioY complexes, which were expressed in *E. coli*, did not have biotin bound after they had been purified by nickel-Sepharose and size-exclusion chromatography.

For EcfAA’T-ThiT complexes yet another method was used to determine whether substrate (in this case thiamin) was bound to the purified complexes. ThiT migrates more slowly on SDS-PAGE when it has thiamin bound (figure 6.5). The change in migration behavior can also be observed when thiamin is added to ThiT that is already in SDS-sample buffer. ThiT probably only denatures slowly in the denaturating SDS-sample buffer. ThiT can be completely unfolded when it is boiled for 5 min in SDS-sample buffer, then only the lower band is visible. Probably the upper band is not (completely) unfolded ThiT that still has thiamin bound.
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Figure 6.5. Samples of purified apo-ThiT purified from *L. lactis* in SDS-sample buffer without (two lanes on left) or with (two lanes on right) thiamin added and analyzed by SDS-PAGE. ThiT with thiamin added runs as two bands. One at the same position as apo-ThiT and the other migrating more slowly and thus at a higher position. The band that migrates more slowly through the gel is of ThiT that has thiamin bound and is probably not completely unfolded.

Figure 6.6. EcfAA'T-ThiT complexes purified from *E. coli* grown on medium with thiamin, without (left lane) or with (right lane) thiamin added before adding sample buffer and loading on SDS-PAGE.

We analyzed EcfAA'T-ThiT complexes expressed in *E. coli* grown on 2xTY medium (which contains thiamin) on SDS-PAGE. ThiT from the EcfAA'T-ThiT complexes only showed the lower (unliganded) ThiT band, unless thiamin was added to the buffer (figure 6.6).

The observation that the isolated EcfAA'T-RibU, EcfAA'T-BioY and EcfAA'T-ThiT complexes did not have substrate bound even though they were expressed in cells growing on 2xTY medium (which contains riboflavin, biotin and thiamin), indicates that the affinity of the complexes for the tested substrates is not as high as for the S-components alone, at least not under the used purification conditions. To further study substrate binding to these complexes tryptophan fluorescence studies were conducted. Such measurements previously showed that RibU, ThiT and BioY have a high affinity for respectively riboflavin (K_d = 0.6 nM (Duurkens et al., 2007)), thiamin (K_d = 0.1 nM (Erkens & Slotboom, 2010)) and biotin (K_d = 0.3 nM, chapter 4). In RibU and ThiT most of the tryptophan fluorescence was quenched when substrate was added (Duurkens et al., 2007; Erkens & Slotboom, 2010). In the case of BioY a blueshift of the tryptophan fluorescence as well as quenching was observed when biotin was added (chapter 4).
Figure 6.7. Tryptophan fluorescence of EcfAA'T-BioY (A) or BioY only (B) before (black line) or after (grey line) the addition of biotin. In both cases a blueshift can be observed.

The EcfAA'T-S-component complexes that were studied here have one extra tryptophan in the S-component because of the STREPII-tag (WSHPQFEK) and seven additional tryptophan residues from the EcfAA'T complex (2 in EcfA, 1 in EcfA’ and 4 in EcfT). Therefore, the signal-to-background ratio was expected to be lower. Nonetheless, changes in the tryptophan fluorescence upon substrate addition were still observed for EcfAA'T-BioY complexes (figure 6.7), but the interpretation of the data was difficult. The tryptophan fluorescence of the complexes was not stable over time, possibly caused by aggregation of the complexes in the cuvette (also observed for EcfAA'T-RibU and EcfAA'T-ThiT complexes). In addition, we also intended to study substrate binding in different conformational states of the protein complex by adding nucleotides such as ATP. But since ATP also absorbs at the excitation wavelength (280 nm) signals became rather low and noisy. Additional problems came from the substrate in the case of thiamin and riboflavin, which also absorb substantially at 280 nm when used at high concentrations. All in all: no conclusive data could be obtained for the EcfAA'T-S-component complexes using the intrinsic tryptophan fluorescence.

To study riboflavin binding to EcfAA'T-RibU complexes also the fluorescence of riboflavin could be used. When riboflavin is titrated into a buffer solution the fluorescence increases proportionally with the concentration. In contrast, when riboflavin is titrated into a solution containing detergent-solubilized, and substrate-free (apo-)RibU the fluorescence of the substrate is initially quenched because it binds to the protein (Duurkens et al., 2007). When RibU is saturated with substrate,
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Figure 6.8. Titration of riboflavin into a buffer solution (black circles) or a solution with detergent-solubilized substrate-free RibU at a concentration of \( \sim 150 \text{ nM} \) (white circles).

Further titration will result in a proportional increase of the fluorescence and the increase at each step is the same as in buffer (figure 6.8) (Duurkens et al., 2007).

This method was used for the purified EcfAA'T-RibU complexes in detergent solution, without any nucleotides (figure 6.9a), supplemented with 5 mM MgADP (figure 6.9b) or 5 mM MgATP (figure 6.9c). When riboflavin was titrated into a solution with detergent-solubilized EcfAA'T-RibU complexes, riboflavin fluorescence was substantially quenched, indicating that the riboflavin was bound. When 1 mM MgADP was present in the EcfAA'T-RibU solution, the quenching was slightly larger and when MgATP was present the quenching was much larger. This indicates that there was higher affinity binding or that there were more binding sites available in the presence of MgATP. However, the quenching did not saturate in any of the conditions (i.e. the lines did not become parallel in figure 6.9a-c as in figure 6.8, and no plateau was reached in figure 6.9d). This makes it impossible to fit the data from figure 6.9d, via the described methods. When riboflavin fluorescence in EcfAA'T-RibU complexes was measured at the end of a titration without the addition of further riboflavin, it was observed that the fluorescence decreased over time. This decrease is probably due to instability of the complexes. Possibly aggregates are formed which scatter the light. The instability of the riboflavin signal, probably due to aggregation of the EcfAA'T-RibU complexes, makes it impossible to calculate reliable \( K_d \) values.
Figure 6.9. Titration of riboflavin into a buffer solution (black circles) or a solution with detergent-solubilized EcfAA'T-RibU (white circles). Both the buffer and the protein solution contained no nucleotides (A), 5 mM MgADP (B) or 5 mM MgATP (C). In D the differences are shown between the fluorescence of riboflavin added to a buffer solution or to a solution with detergent-solubilized EcfAA'T-RibU: without nucleotides (black circles), with 5 mM MgADP (white circles) or with 5 mM MgATP (black triangles).

6.4.3 Effect of substrate and/or nucleotides addition

In the previous chapter, we showed that EcfAA'T-ThiT and EcfAA'T-NiaX complexes probably dissociate during the transport cycle. Here, we tried to find conditions that would lead to the dissociation of ThiT from the purified EcfAA'T-ThiT complex by adding substrate and/or nucleotides. The EcfAA'T-ThiT peak fractions from size-exclusion chromatography were analyzed by SDS-PAGE (figure 6.10). All fractions contain the EcfAA'T proteins and ThiT. The complex thus does not fall apart in detergent solution under the conditions that we tested.

Interestingly EcfAA'T-ThiT appeared to have some thiamin bound when it was purified in the presence of MgATP or MgADP, while no thiamin was added during the purification. This could indicate a higher binding affinity. However, since the additions were done after the elution from the nickel-column, the thiamin must have remained bound during the nickel purification steps, which did not seem to
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Figure 6.10. EcfAA'T-ThiT peak-fractions after a run on a gel filtration column with the indicated additions to the buffer and the loaded sample. A Western blot using STREPII antibodies (bottom panel) verified that ThiT remained bound to the EcfAA'T-complex, no matter which components were added.

be the case (figure 6.6). Possibly the additions of nucleotides and/or magnesium had an (aspecific) effect on the migration behavior of ThiT on SDS-PAGE.

When substrate (thiamin) was added prior to the size-exclusion chromatography run and also to the buffer used in the chromatography step, the EcfAA'T-ThiT protein complex no longer eluted as a sharp peak (figure 6.11). Most of the EcfAA'T-ThiT complex eluted earlier: around 10 mL instead of at 11.7 mL and simultaneously a lot of protein seemed to have been lost (possibly in the centrifugation step before loading on the column, which is done to remove large aggregates). This indicates that EcfAA'T-ThiT aggregated when thiamin was added.

When the fractions from the size-exclusion chromatography runs of EcfAA'T-ThiT with different additions of substrate and/or nucleotides were loaded on SDS-PAGE, it was confirmed that EcfAA'T-ThiT eluted earlier and as a broader peak from the size exclusion column when thiamin was added (figure 6.12).

This experiment also was performed for other EcfAA'T-S-component complexes (EcfAA'T-NiaX and EcfAA'T-RibU were used) and consistently the aggregation was observed when substrate was added. Sometimes it appeared to be even more
pronounced if MgATP was also present (figure 6.12). The apparent instability of the complex in the presence of substrate might also explain the problems with the fluorescence titration measurements (figure 6.9). The results may indicate that the EcfAA'T-S-component complexes form oligomers during the translocation cycle, although it is also possible that the complexes become less stable in detergent solution due to a conformational change that is induced by substrate.
6.4.4 EcfAA’T-S-component complex stability in various buffers

To determine the ATPase activity of purified ECF complexes we made use of a method that determines free phosphate concentrations. For this assay, the media must be phosphate-free and therefore the previously used potassium phosphate buffer was not suitable. During the search for a buffer that could be used to purify the EcfAA’T-S-complexes but that did not contain phosphate, we used the EcfAA’T-NiaX complex, which gives the highest yield and the most symmetric peak on a size-exclusion column. It was found that the EcfAA’T-NiaX complex was not stable in commonly used buffers such as HEPES, MOPS, TRIS or imidazol buffer but maintained intact in ammonium acetate or ammonium bicarbonate buffer (all at pH 7.5 and at 50 mM, figure 6.13 and 6.14).

Figure 6.13. The elution profile of EcfAA’T-NiaX on a size-exclusion column in the following buffers: TRIS (purple line), MOPS (orange line), imidazol (yellow line) or HEPES (green line). EcfAA’T-NiaX complexes (that normally elute around 11.7 mL) were not stable in any of these buffers. The protein that eluted at 15.5 mL was not EcfAA’T-NiaX but a contaminant.
Figure 6.14. The elution profile of EcfAA'T-NiaX on a size-exclusion column in the following buffers: potassium phosphate (yellow line), ammonium bicarbonate (ABC, purple line) or ammonium acetate (orange line). EcfAA'T-NiaX complexes eluted around 11.7 mL.

6.4.5 ATPase activity

The ATPase activity of wildtype EcfAA'T-ThiT complexes was compared to complexes with single and double mutations in the ATPases or nucleotide binding proteins. EcfA and/or EcfA' were mutated by a substitution of the glutamate at the end of the Walker B motif for a glutamine.

The substitution of the glutamate at the end of the Walker B motif for a glutamine in EcfA and/or EcfA' had a large effect on the ATPase activity of the EcfAA'T-ThiT complex (figure 6.15). The double mutant showed no ATPase activity, indicating that the glutamate at the end of the Walker B motif is required for ATP hydrolysis. Both single mutants, with only one of the two ATPase domains mutated, showed roughly 40% activity compared to wildtype.
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Figure 6.15. ATPase activity of EcfAA’T-ThiT complexes with wildtype EcfA and EcfA’ (wildtype), mutated EcfA (EcfA:E166Q), mutated EcfA’ (EcfA’:E170Q) or with both ATPase domains mutated (double mutant). The background level of phosphate in the buffer was also measured. Wildtype activity is set to 100% and error bars show the range of 2 measurements.

6.5 Discussion

In this chapter, we show that *E. coli* cells expressing EcfAA’T-BioY or EcfAA’T-PanT bind radiolabeled substrate (780-900 molecules per cell) and that similar numbers are obtained when only the S-components are expressed. In both cases the amount of radioactivity associated with the cells reduced to background levels when unlabeled substrate was added in excess. The release was rapid for BioY and PanT, irrespective of whether the ECF module was co-expressed.

Also for EcfAA’T-RibU and RibU expressing cells the number of [3H]riboflavin molecules that were initially bound were comparable (∼2000 riboflavin molecules per cell). This number rapidly decreased in cells expressing RibU only when unlabeled riboflavin was added, while for EcfAA’T-RibU this decrease went slower and 700-1100 molecules remained bound. This might indicate that there was some transport of riboflavin by EcfAA’T-RibU.

EcfAA’T-BioY2 expressing cells did bind/transport more biotin molecules than BioY2 expressing cells (∼420 and ∼300 molecules/cell, respectively). After the addition of unlabeled biotin, BioY2 expressing cells instantly decreased [3H]biotin binding to background levels. For EcfAA’T-BioY2 expressing cells, the amount
Table 6.1. Differences in the membrane composition between \textit{L. lactis} and \textit{E. coli}.
(Bishop \& Bermingham, 1973; Veld et al., 1991; Spitsmeister et al., 2010; Driessen et al., 1988)

<table>
<thead>
<tr>
<th>Lipid composition</th>
<th>\textit{E. coli} (Gram-negative bacterium)</th>
<th>\textit{L. lactis} (Gram-positive bacterium)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>77-78% phosphatidyl ethanolamine (PE),</td>
<td>32% diphosphatidylglycerol (cardiolipin, CL),</td>
</tr>
<tr>
<td></td>
<td>14% diphosphatidylglycerol (cardiolipin, CL),</td>
<td>23% dihexosyl diglyceride,</td>
</tr>
<tr>
<td></td>
<td>8-9% phosphatidylglycerol (PG)</td>
<td>22% glycerophosphoglycolipid,</td>
</tr>
<tr>
<td>Fatty acid composition</td>
<td>~40% palmitate (C16:0),</td>
<td>~31% palmitate (C16:0),</td>
</tr>
<tr>
<td></td>
<td>~30% palmitoleate (C16:1),</td>
<td>~21% lactobacillic acid (C19Δ),</td>
</tr>
<tr>
<td></td>
<td>~19% oleate (C18:1),</td>
<td>~17% oleate (C18:1),</td>
</tr>
<tr>
<td></td>
<td>~8% C17:0Δ,</td>
<td></td>
</tr>
<tr>
<td></td>
<td>~4% myristate (C14:0),</td>
<td></td>
</tr>
<tr>
<td></td>
<td>~1% C15:0, C18:0, C19:0Δ</td>
<td></td>
</tr>
</tbody>
</table>

of $[^3]$H]biotin also decreased but initially stayed above background. However, 4 min after addition of non-labelled substrate the $[^3]$H]biotin levels had decreased to background levels.

ABC importers usually do not operate in the reverse direction under \textit{in vivo} conditions, and the fast decrease in radioactivity levels indicates that the substrate was bound to the outside of the cell (presumable to the S-component) and was not transported to the inside of the cell in most cases. Possibly there was some transport in the case of EcfAAT-RibU and EcfAAT-BioY2, but the large amount of substrate binding makes it difficult to quantify. Apparently, the interactions of the ECF module with the S-components BioY and PanT was not sufficient to support transport when present in the heterologous \textit{E. coli} host. Interactions between EcfT and the S-component in the lipid bilayer are required for activity (chapter 1, 4 and (Erkens et al., 2011)). The membrane composition, which differs between \textit{E. coli} and \textit{L. lactis} (see table 6.1), could have an effect on this interaction. Nonetheless, the same ECF module from \textit{L. lactis} functions in \textit{E. coli} together with ThiT and NiaX, possibly because of stronger interactions between the EcfAAT and these S-components. EcfAAT-RibU and EcfAAT-BioY complexes were shown to be active when purified from \textit{E. coli} and reconstituted in \textit{E. coli} polar lipids and egg phosphatidylcholine (chapter 3 and 4).

In chapter 5, it was observed that the S-components ThiT and NiaX form transient complexes with the ECF module. It was also observed that these S-components bound stronger to the ECF module when they had substrate bound than when
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there was no substrate present. The purified EcfAAT-BioY, EcfAAT-RibU and EcfAAT-ThiT complexes did not have substrate bound. Possibly the substrate was lost during the purification procedure and the EcfAAT-S-component complexes remained associated, because they were located in the detergent micelle instead of the membrane bilayer. The detergent micelle may force the EcfAAT and the S-component to stay together. Consistently, we were unable to let EcfAAT-ThiT complexes fall apart by adding nucleotides and/or substrate. S-component exchange on the EcfAAT-S-component complexes is most probably prevented by the detergent micelle.

It was difficult to study substrate binding via fluorescence measurements, because the EcfAAT-S-component complexes had the tendency to aggregate in detergent micelles, especially when substrate was present. However, from the preliminary data presented here, it appears that the addition of MgATP had a strong effect on riboflavin binding to the EcfAAT-RibU complex. High-affinity riboflavin binding was observed when MgATP was added to the EcfAAT-RibU complex. When no nucleotides or MgADP were present, the affinity seemed to be lower and/or less binding sites were accessible. Although binding sites may become inaccessible and/or the binding affinity may be lowered because of a change in conformation, solid conclusions are not possible at this point. We considered trying other methods such as isothermal titration calorimetry (ITC), but this will be difficult because of the low protein yields, and also here the aggregation is likely a problem. It would be interesting to repeat the fluorescence binding studies with EcfAAT-S-component complexes reconstituted in nanodiscs. Proteins incorporated into nanodiscs are embedded in a membrane bilayer, which could improve the stability and allow structural changes to take place without aggregation.

ABC transporters couple ATP hydrolysis to substrate translocation. No ATPase activity was found in a double mutant, where both ATPase domains were mutated by substituting the glutamate at the end of the Walker B motif for a glutamine. This result indicates that the glutamate, which is proposed to be the general base that polarizes the attacking water molecule (introduction and (Davidson et al., 2008)), is indeed essential for ATPase activity. Similar results were obtained for various other ABC transporters (Orelle et al., 2003; Davidson et al., 2008; Geourjon et al., 2001; Moody et al., 2002). The single ATPase domain mutations in EcfAAT-ThiT remained ∼40% active. For other ABC transporters, the effect of single site mutations seems to differ. For the histidine transporter ∼66% activity was found for a single ATPase domain mutation (where the histidine from the H-loop, was...
mutated into an arginine) (Nikaido & Ames, 1999), while in the maltose transporter only 6% activity remained when the lysine of the Walker A was mutated to an asparagine in one of the two ATPase domains, and 12% when the histidine of the H-loop was mutated to an arginine in one of the two ATPase domains (Davidson & Sharma, 1997).

For many ABC transporters and also for the EcfAA'T-S-component complexes studied here, a high basal activity is found in detergent solution without substrate addition. Possibly ATP hydrolysis is uncoupled from the transport process because of the lack of a lipid bilayer. Therefore, it would be of interest to measure the ATPase activity of the different EcfAA'T-S-component complexes with and without substrate in nanodiscs or liposomes. For the ABC transporter MsbA, it has been shown that ATPase activity is influenced by the composition of the bilayer (Kawai et al., 2011). In nanodiscs the proteins are inserted into a lipid bilayer, but both sides of the membrane are accessible so that the addition of nucleotides and substrate can be varied directly and simultaneously. The lipid environment possibly reduces the basal ATPase activity in the absence of substrate. For ECF-type ABC importers futile cycling in absence of transport may also be kept low by dissociation of the ECF module and the S-component complex in absence of substrate. It will be interesting to see if the ATPase activity of the ECF module depends on which S-component is bound.