Subunit composition of an ECF-type biotin transporter analyzed in living bacteria

Friedrich Finkenwirth, Olivia Neubauer, Julia Gunzenhäuser, Janna Schoknecht, Silvia Scolari, Martin Stöckl, Thomas Korte, Andreas Herrmann, Thomas Eitinger

► To cite this version:

Friedrich Finkenwirth, Olivia Neubauer, Julia Gunzenhäuser, Janna Schoknecht, Silvia Scolari, et al.. Subunit composition of an ECF-type biotin transporter analyzed in living bacteria. *Biochemical Journal*, 2010, 431 (3), pp.373-380. 10.1042/BJ20100813 . hal-00525046

HAL Id: hal-00525046

<https://hal.science/hal-00525046>

Submitted on 11 Oct 2010

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Subunit composition of an ECF-type biotin transporter analyzed in living bacteria

Friedrich FINKENWIRTH^{*1}, Olivia NEUBAUER^{*1}, Julia GUNZENHÄUSER^{†2}, Janna SCHOKNECHT^{*}, Silvia SCOLARI[†], Martin STÖCKL^{†3}, Thomas KORTE[†], Andreas HERRMANN[†], and Thomas EITINGER^{*4}

^{*}Institut für Biologie/Mikrobiologie, [†]Institut für Biologie/Molekulare Biophysik; Humboldt-Universität zu Berlin, 10115 Berlin, Germany

Short title: Subunit composition of an ECF transporter

¹ These two authors contributed equally to the work.

² Present address: EPFL IPSB LEB BSP/Cubotron 425, 1015 Lausanne, Switzerland.

³ Present address: Biophysical Engineering Group, MESA+ Institute for Nanotechnology, University of Twente, P.O. Box 217, 7500 AE Enschede, The Netherlands.

⁴ To whom correspondence should be addressed (email thomas.eitinger@cms.hu-berlin.de).

Abbreviations used: DDM, dodecyl- β -D-maltoside; ECF, energy-coupling factor; FRET, Förster resonance energy transfer; mCerulean, monomeric Cerulean; mYFP, monomeric yellow fluorescent protein; TC, transporter classification system;

SYNOPSIS

BioMNY, a bacterial high-affinity biotin transporter, is a member of the recently defined class of energy-coupling factor (ECF) transporters. These systems are composed of ABC ATPases (represented by BioM in the case of the biotin transporter), a universally conserved transmembrane protein (BioN), and a core transporter component (BioY) in unknown stoichiometry. The quaternary structure of BioY, which functions as a low-affinity biotin transporter in the absence of BioMN, and of BioMNY was investigated by a FRET approach using living recombinant *E. coli* cells. To this end, the donor-acceptor pair of Cerulean and yellow fluorescent protein, respectively, were fused to BioM, BioN and BioY. The fusion proteins were stable and the protein tags did not interfere with transport and ATPase activities. Specific donor-acceptor interactions were characterized by lifetime-based FRET spectroscopy. The data argue for an oligomeric structure of the solitary BioY core transporter and oligomeric forms of BioM and BioY in BioMNY complexes. We surmise that oligomers of BioY are the functional units of the low- and high-affinity biotin transporter in the living cell. Beyond its relevance for clarifying the supramolecular organization of ECF transporters, our work demonstrates the general applicability of lifetime-based FRET to living bacteria.

Key words: FRET, fluorescence lifetime, ABC transporter, vitamin uptake, ECF transporter

INTRODUCTION

Energy-coupling factor (ECF) transporters are a recently identified and abundant class of prokaryotic high-affinity import systems for vitamins, transition metal ions, and intermediates of salvage pathways ([1], reviewed in ref. [2]). Many pathogenic bacteria with restricted biosynthetic capacities rely on vitamin uptake from the host by ECF importers. These transporters share a common architecture consisting of pairs of ATP-binding cassette-containing (ABC) ATPases (A components), a universally conserved transmembrane protein (T component), and a transmembrane substrate-capture protein (S component or core transporter) in unknown stoichiometry. A- and T units form a module called – for historical reasons – the "energy-coupling factor". S components are highly diverse small integral membrane proteins with molecular masses of ~20 kDa. They bind their substrates with dissociation constants in the low nanomolar or picomolar range [3-5].

ECF systems resemble ABC transporters because both classes of transporters rely on ABC ATPases. However, they differ in the modular composition and in the requirement for extracytoplasmic solute-binding proteins which are essential components of canonical ABC importers but are absent from ECF systems. Based on genomic localization of their genes and usage of a dedicated or shared energy-coupling module, ECF systems fall into two subclasses. The A-, T- and S components of subclass I systems form a functional unit and are encoded in operons. In the case of subclass II systems, the EcfA1A2T module is encoded on one operon but the genes for the various S components are scattered around the chromosome. Notably, those S units, although unrelated on the amino acid sequence level, compete for copies of the same EcfA1A2T module to form functional holotransporters [1, 6].

BioMNY of the α -proteobacterium *Rhodobacter capsulatus* represents the prototype of subclass I ECF transporters. It can be produced in a functional state and in reasonably large quantities in recombinant *E. coli* cells. Previous experiments have shown that the solitary BioY (S component) containing six predicted transmembrane domains acts as a high-capacity biotin transporter in the absence of its A- (BioM) and T- (BioN) units. High-affinity transport, however, is dependent on a functional BioM ATPase [7]. A role in complex stabilization and intersubunit signaling has recently been assigned to BioN [6]. Although these studies gave initial insight into individual functions of A-, T- and S units, the structural organization as the basis of solute recognition and translocation remains to be clarified. In particular, the oligomeric states of the solitary BioY and the BioMNY holotransporter have not yet been elucidated.

To assess interactions among proteins live-cell fluorescence technologies including Förster resonance energy transfer (FRET) have become powerful tools. However, while they are widely applied to eukaryotic cells (see refs. [8-10] for recent reviews), FRET-based approaches have not found a broader application to living prokaryotic cells. Usually, FRET measurements on cells are done by steady-state monitoring of the intensity of the donor before and after bleaching of the acceptor. However, bleaching of the acceptor may be incomplete interfering with correct measurement of FRET [11]. An alternative way to assess FRET is the measurement of the donor fluorescence lifetime circumventing acceptor bleaching [12]. Here, we report that lifetime-based FRET is applicable to living bacteria. Fusing Cerulean as the donor and yellow fluorescent protein as the acceptor to BioM, BioN, and BioY, preserving their functional activity, we were able to detect specific protein-protein interactions providing insight into the subunit composition of the BioMNY system in the living bacterial cell.

MATERIALS AND METHODS

Bacterial strains

E. coli BL21 with plasmid pLacI-RARE2, encoding a Lac repressor and seven tRNAs for rare codons, was used as the host for expression of tagged and untagged biotin transporter genes. To determine FRET, the donor and acceptor were produced from two plasmids each derived from vector pBluescriptIIKS+. The two plasmids contain a *lac* promoter and a ribosomal binding site, and are identical to a large extent except for the antibiotic resistance gene [6, 7]. mCerulean fusions were produced from streptomycin resistance-conferring plasmids and mYFP fusions from ampicillin resistance-conferring variants. For biotin-uptake experiments recombinant cells of the intrinsically biotin transport-deficient *E. coli* strain S1039 containing the LacI-encoding plasmid pFDX500 and a BioY-encoding plasmid were used as described in ref. [7].

Standard molecular biological techniques

Plasmids encoding the fluorescent proteins mCerulean [13] and mYFP [14] were kindly provided by Stephanie Engel (Berlin, Germany). mCerulean and mYFP coding sequences were amplified using Pfu proofreading DNA polymerase (Invitrogen) and primers 5'-GCGAGATCTGCCATGGTGAGCAAGGGCGAG-3' and 5'-GCGCCATGGCAGATCTCTTGTACAGCTCGTCCATGC-3'. The products were subsequently treated with Taq polymerase to generate A-overhangs and inserted into vector pGEM-T, and the DNA sequences were verified by nucleotide sequencing.

Confocal fluorescence microscopy

Recombinant *E. coli* BL21 cultures were diluted 1:100 in 10 ml LB medium and grown for two hours at 37 °C under shaking. Upon addition of 1 mM IPTG to induce expression of genes under control of the *lac* promoter, incubation was continued for another 2.5 hours. Cells were harvested and resuspended in 500 µl 35 mM sodium potassium phosphate buffer (pH 7.0). For labeling with the membrane marker *N*-(3-triethylammoniumpropyl)-4-(6-(4-(diethylamino)phenyl)hexatrienyl)pyridinium dibromide (FM4-64; Invitrogen), 15 µl of the cell suspension was mixed with 15 µl of the phosphate buffer and 5 µl of an FM4-64 solution (20 µg/ml). After incubation for 15 min at room temperature in the dark, 10 µl of the stained cells were mixed with 10 µl of a 2 % low-melting agarose solution. Samples were subjected to fluorescence microscopy using an Olympus FV1000 confocal laser microscope equipped with a UPlanSApo 60x/1.35 oil immersion objective. Pictures were processed with FLUOVIEW (Olympus) and COMBINEZM [15, 16].

Lifetime-based FRET spectroscopy

Cultures (10 ml) grown essentially as described for fluorescence microscopy were harvested at an OD₅₇₈ ~1.8, washed with 35 mM sodium potassium phosphate buffer (pH 7.0) and concentrated 2.5-fold. 200 µl aliquots were subjected to the assays. Fluorescence lifetimes were measured at 25 °C using a PicoQuant FluoTime 200 spectrometer. The samples were excited with pulsed laser light (440 nm; 8 MHz) and mCerulean-based fluorescence intensity

decays with 10^4 counts were recorded at 475 nm by means of time-correlated single photon counting (TCSPC). Scattered light at wavelengths below 460 nm arising from the bacterial cells was filtered out. The instrument response function (IRF) was recorded for each experimental setup using phosphate buffer. Seven intensity decays were collected and fitted globally using a nonlinear least square iterative reconvolution fitting procedure (FLUOFIT, PicoQuant, Germany):

$$I(t) = \int_{-\infty}^t \text{IRF}(t') \left(\sum_{i=1}^n \alpha_i e^{-\frac{t-t'}{\tau_i}} \right) dt'$$

All intensity decays were fitted to a biexponential function with two fluorescence lifetimes [14], τ_1 and τ_2 , being in the order of 1 ns and 3 ns, respectively. Quality of the fit was judged by the χ^2 value, the distribution of residuals and the autocorrelation function of residuals. A mean lifetime τ was determined by weighting τ_1 and τ_2 according to their amplitudes. This procedure was applied 4 times, and mean lifetime values of those four determinations were averaged representing one sample $\pm$ its standard deviation (Samples $\pm$ SD). The left side in each part of Figures 4 and 5 (see later in the results section) refers to those individual determinations for biological and technical replicates. τ values of independent samples were averaged and are given $\pm$ the standard error of the mean (Means $\pm$ SEM) in the middle part of Figures 4 and 5.

Biotin uptake assay

Biotin uptake of recombinant *E. coli* S1039 was assayed as described in ref. [7] with the following modifications: Cells were grown in ampicillin-containing LB medium, washed in glucose-free buffer and resuspended in the same buffer containing 4 nM [^3H]biotin at an optical density (OD_{578}) of 0.2, and incubated for four hours at 37 °C under shaking. Samples (0.5 ml) were filtered, the filters were washed and filter-bound radioactivity was quantitated by liquid scintillation counting.

Purification of biotin transporter complexes and assay of ATPase activity

Recombinant *E. coli* BL21 (800 ml cultures) was grown in LB medium in the presence of 1 mM IPTG, cells were harvested at an $\text{OD}_{578} \sim 2$, washed in 35 mM sodium/potassium phosphate buffer (pH 7.0) and lysed by three passages through a French pressure cell upon addition of protease inhibitors. Membranes were collected by ultracentrifugation and solubilized in 50 mM Tris-HCl (pH 8.0) containing 300 mM NaCl, 5 % glycerol, 20 mM imidazole and 2 % (w/v) dodecyl- β ,D-maltoside (DDM). Non-solubilized material was pelleted by ultracentrifugation. The supernatant was mixed with Ni-NTA matrix (1 ml bed volume) and the mixture was incubated overnight at 4 °C with gentle shaking. Upon transfer into an empty column, the samples were washed with 60 ml 50 mM Tris-HCl (pH 7.5) containing 300 mM NaCl, 5 % glycerol, 75 mM imidazole and 0.05 % (w/v) DDM. Bound protein was eluted with 2 ml of the buffer containing 500 mM imidazole. The samples were desalted by passage through PD MiniTrap G-25 columns (GE Healthcare) and subjected to SDS-PAGE and Western blotting, and to ATPase assays. ATPase activity was determined as described [7].

RESULTS

Construction of mCer- and mYFP-tagged components of the biotin transporter

Fusions of enhanced cyan fluorescent protein (ECFP, as the donor) and enhanced yellow fluorescent protein (EYFP, as the acceptor) are frequently used in FRET experiments (reviewed in ref. [10]). Cerulean is a variant of ECFP with increased brightness and thus, it is superior to ECFP as a FRET donor [13]. Several fluorescent proteins intrinsically tend toward oligomerization. This property interferes with protein-protein interaction studies by FRET of target proteins tagged with the donor and the acceptor. The A206K replacement is known to prevent intrinsic oligomerization ([12], reviewed in ref. [17]). Here we fused monomeric variants with the A206K replacement of Cerulean (mCer) and enhanced yellow fluorescent protein (mYFP) to the three components of the biotin transporter in order to analyze protein-protein interactions by FRET. The strategy for construction of the fusions is explained in detail in the online supplement.

Localization and stability of the fluorophore-tagged proteins

The ABC ATPase BioM is a soluble protein in the absence of BioN and BioY, but is assembled in membrane-bound BioMN and BioMNY complexes of unknown stoichiometry in the presence of the integral membrane proteins BioN or BioN+BioY, respectively [7]. In a series of experiments we analyzed by confocal fluorescence microscopy whether fusions of mCer affect cellular localization of the solitary BioM, the solitary BioY, the BioMN subcomplex and the BioMNY holotransporter complex. The results are shown in Figure 1. As expected, production of BioM-mCer in the absence of its partners resulted in fluorescence signals homogeneously distributed in the cell. In cells co-producing BioN+BioY, however, the fluorophore-tagged BioM was essentially visible in the cell envelope, as indicated by co-localization with the membrane marker FM4-64. These observations demonstrated that fusion of mCer did not alter the property of BioM to form membrane-bound complexes with its partners. Likewise, attachment of mCer to BioY and BioN did not interfere with membrane insertion of the two proteins. Tagged BioN required co-production with BioM, because synthesis of the solitary BioN is not tolerated by *E. coli* cells. As a control in FRET experiments (see below), we used a bacterial Co^{2+} transporter (a member of the NiCoT family, TC 2.A.52) that is unrelated to ECF systems and is contained in large quantities in *E. coli* upon recombinant production [18]. As expected, the mYFP-tagged NiCoT protein was identified selectively in the membrane by fluorescence microscopy (Figure 1).

Stability of the fusion proteins is a prerequisite for assigning fluorophore-fluorophore interactions to ensembles of the target proteins. Therefore, we tested robustness of N-terminally and C-terminally tagged BioM and BioY and of C-terminally tagged BioN in recombinant *E. coli* cells. Cell suspensions were lysed in SDS-containing sample buffer and aliquots were subjected to SDS-PAGE and Western blotting. As shown in Figure 2, the bands correlated with the molecular masses of the fusion proteins (BioN-mCer-FLAG, 50.7 kDa; His₁₀-BioY-mCer-FLAG, 49.3 kDa; His₁₀-BioM-mCer-FLAG, 55.2 kDa; His₁₀-mYFP-BioM-FLAG, 55.4 kDa; His₁₀-mYFP-BioY-FLAG, 49.4 kDa; His₁₀-mCer-BioM, 53.9 kDa). In no case were degradation products or the free fluorescent protein (~27 kDa) visible, thus confirming the stability of the fusion proteins.

Functional state of tagged biotin transporter proteins

To examine whether fusion of a fluorescent protein affects the biological function of the biotin transporter components, we compared biotin-uptake activity of wild-type and tagged BioY, analyzed formation of protein complexes in the presence of a tagged component, and measured ATPase activity of the holotransporter complexes. The results are shown in Figure 3. Biotin-uptake assays with recombinant *E. coli* cells provided direct evidence for intactness of tagged BioY. As illustrated in Figure 3A, N-terminally and C-terminally tagged BioY was almost unaffected by the attached fluorophore and mediated biotin transport.

In the next series of experiments, we investigated the effect of fluorophore tags on stability of the BioMN module and the holotransporter. The results of purification studies are shown in Figure 3B. BioMN (Figure 3B, lanes a-f) and BioMNY (lanes g-r) complexes were purified by affinity chromatography via the His-tag on BioM and the subunit composition was analyzed by SDS-PAGE and subsequent Coomassie staining (left lane in each three-lane panel of Figure 3B) or Western blotting with anti-FLAG (middle lane) or anti-oligo-His (right lane) antibodies. These experiments demonstrated that fusion of mCer to the C-terminus of BioN (lanes d-f) did not interfere with formation of BioMN complexes, and neither fusion of mCer to the N-terminus of BioM (lanes m-o) nor the C-terminus of BioY (lanes p-r) affected the tripartite composition of BioMNY complexes. The tripartite BioM_{K42N}NY complex with an inactivating Walker A site mutation in BioM (lanes j-l) was purified as a negative control for subsequent ATPase assays.

We have shown in two previous studies that ATPase activity is a reliable indicator of intactness of tripartite BioMNY complexes [6, 7]. Specifically, it was evident that the solitary BioM protein does not mediate ATP hydrolysis. Therefore, we tested this activity of complexes harboring mCer-tagged BioM or mCer-tagged BioY. Figure 3C demonstrates that none of the modifications markedly altered ATPase activity. The results indicate that the presence of a fluorescent protein does not interfere with the physical and functional states of biotin transporter complexes.

Oligomeric states of BioM and BioY in living cells

We used a FRET-based approach to unravel the multi-subunit composition of the BioMNY system in living bacteria. Specifically, we addressed the question whether multiple copies of certain subunits are detectable by this technique. Plasmids encoding fusions of mCer and mYFP to components of the biotin transporter were co-transformed into *E. coli* cells and the cell suspensions were analyzed by lifetime based FRET. Initially, we measured FRET by fluorescence lifetime imaging microscopy of single cells. This approach is hampered by a rather low number of fluorescence counts. Therefore, knowing the intracellular localization of constructs (see also fluorescence microscopy), we employed lifetime measurements of cell suspension by spectroscopy. The first series of experiments focused on the BioM ATPase. Since dimerization is a requirement for ATP hydrolysis by ABC ATPases (see ref. [19] for a review), it is conceivable that BioM is a dimer in functional BioMNY complexes. The presence of mYFP-tagged BioM (as a FRET acceptor) led to significant reduction of the fluorescence lifetime τ of mCer-tagged BioM (as the FRET donor), provided that BioN and BioY were present in the cells (Figure 4B). As FRET efficiency decreases with the sixth power of the distance, shortening of the donor fluorescence lifetime by the acceptor indicates spatial proximity of the fluorophores in a distance range below 10 nm. This points to an oligomeric state of BioM upon complexing with BioN and BioY. Notably, FRET was observed for both N- and C-terminally mYFP-tagged BioM which may provide important information how BioM becomes arranged in the transporter complex. In contrast, no decrease

of τ of the donor was observed for pairs of BioM variants that are randomly distributed in the cytosol in the absence of BioNY (Figure 4A).

FRET was observed between donor-tagged BioN and acceptor-tagged variants of BioM (Figure 4C). This result is in agreement with the biochemical experiments that identified stable complexes composed of BioM and BioN. Control experiments (right panels in Figure 4) confirmed the reliability of our FRET approach: neither untagged BioM (Figure 4B) nor mYFP-tagged NiCoT (Figure 4A, B and C) caused a reduction of τ of the donor. The latter result excluded the possibility that FRET observed for the tagged components of the biotin transporter is due to intrinsic dimerization of the GFP-derived fluorophores.

The focus of the next series of experiments was on oligomeric state analysis of the core transporter BioY. The supramolecular organization in living cells is not known for any core transporter of ECF systems. Results of lifetime-based FRET measurements are depicted in Figure 5. FRET was clearly visible for BioY pairs in the absence (Figure 5A) and presence (Figure 5B) of BioMN. Again, control experiments in which acceptor-tagged BioY was replaced by native BioY (Figure 5, right panels) or by NiCoT-mYFP (Figure 5A, right panel) did not detect FRET. The latter finding substantiated the specificity of FRET observed for the pair of tagged BioY proteins, and excluded the possibility that FRET occurs nonspecifically due to overproduction of tagged membrane proteins. Notably, we found an increase of fluorescence lifetime for the BioMNY-mCer when co-produced with nontagged BioY (Figure 5B, right panel). This suggests that the organization of Cerulean, and hence its fluorescence lifetime, is affected when more than one Cerulean is present in the BioMNY transporter complex, a situation that exists in cells producing BioM, BioN and BioY-mCer. An oligomeric composition of BioY would put at least two Cerulean macromolecules in close proximity. In conclusion, our findings suggest that BioY in the living cell has an oligomeric structure in its solitary state as well as in complex with the BioM and BioN proteins.

DISCUSSION

Oligomeric states in the living cell are not known for any ECF transporter. In particular, the supramolecular composition of the core transporters, small transmembrane proteins with molecular masses of ~20 kDa in most cases [1, 2], remains enigmatic. Recently, the oligomeric state of ThiT, the S unit of the subclass II thiamine transporter of *Lactococcus lactis* has been reported. Static light scattering analysis of the protein in detergent solution clearly identified ThiT as a monomer [5]. This finding, however, does not allow clear-cut conclusions on the oligomeric ensemble in the native membrane, since complexes may fall apart in the presence of a detergent. Discrepancies between in vitro and in vivo analyses have been recently exemplified in the case of DcuS of *E. coli*, the membrane-bound histidine kinase of a two-component regulatory system that senses extracellular C₄-dicarboxylates. A combination of chemical cross-linking and intensity-based FRET analyses identified DcuS as a monomer in detergent solution but as an oligomer in living cells [20]. In light of this problem, preliminary results obtained by size-exclusion chromatography-coupled laser light scattering [21] of the solitary BioY in detergent solution, pointing to a monomeric state (D. J. Slotboom, O. Neubauer and T. Eitinger, unpublished result), must be interpreted with caution as regards the oligomeric state of BioY under natural conditions.

In the present study, we used a fluorescence lifetime-based FRET approach to get insight into the supramolecular composition of BioMNY, the prototype of subclass I ECF transporters, under natural conditions, i.e. in living bacterial cells. Fluorescence techniques including FRET can measure interactions between macromolecules in living cells, therefore avoiding the need of detergent solubilization in the case of membrane proteins. However, although several bacterial proteins have been fused to fluorescent proteins and their

localization has been determined, FRET has only rarely been applied to living prokaryotic cells [22-24].

Here, we fused monomeric Cerulean as the donor and monomeric yellow fluorescent protein as the acceptor to BioM, BioN and BioY, and analyzed FRET with various combinations. Upon carefully verifying that the fusion proteins were stable and excluding potential impact of the protein tags on the biological activity of the targets, lifetime-based FRET allowed us to draw three major conclusions: (i) The ABC ATPase BioM is predominantly monomeric in solution, but oligomeric when complexed with the integral membrane proteins BioN and BioY. (ii) Copies of BioY, if present in the absence of BioMN, are localized in close proximity to each other suggesting that the core biotin transporter acts as a dimer or an oligomer. (iii) Likewise, the BioMNY holotransporter contains at least two copies of BioY.

Our results demonstrate that formation of oligomeric protein complexes can be analyzed in living bacteria by lifetime-based FRET spectroscopy, an approach that complements in vitro techniques like the aforementioned light scattering method [21]. Essentially, FRET can be applied to identify protein-protein interactions and to monitor changes that arise from alterations in the affinity between the two proteins or conformational changes of proteins in a complex. In principle, FRET should also provide a method to characterize donor-acceptor stoichiometry in protein complexes. However, assessing stoichiometry has been found to be extremely difficult or even impossible due to the dependence of FRET efficiency of several parameters such as the distance separating the donor-acceptor pair and the relative orientation of the fluorophores to each other. For example, the latter could be different for oligomeric complexes of donor and acceptor. Only under specific conditions, fluorescence microscopy can be used to assess donor-acceptor stoichiometry [25]. Our findings are of general importance on the way to elucidate the mechanism of ECF-type transporters which is still unknown. Knowledge of the mechanism may provide avenues for antibacterial treatment since multiple ECF systems in pathogenic gram-positive bacteria are essential for growth.

The core transporter BioY confers low-affinity biotin-transport activity on recombinant *E. coli* cells in the absence of the BioMN module. Although it cannot be ruled out that BioY interacts with an ECF-related endogenous component of the host, this scenario is unlikely since laboratory strains of *E. coli* K-12 and B lack ECF transporters. Thus, the available data argue for a transporter function of the solitary BioY. Our present data suggest that the molecular basis of this activity is a homooligomer of BioY. This implies that a monomer of BioY with its six predicted transmembrane domains may be insufficient to act as a functional transporter. An oligomeric ensemble of BioY was also found in the high-affinity uptake system BioMNY. Thus, we surmise that the same core of BioY represents the functional transport unit in the low and in the high-affinity system. If core transporters (or S units) are capable of substrate transport in their solitary state, questions on the role of the A1, A2 and T components arise. Within the biotin transporter, A1 and A2 are represented by copies of BioM, and T is represented by BioN. The fact that in the presence of A1A2T the affinity of the transporter for biotin is 50fold increased whereas the maximal velocity is decreased 10fold [7], points to a function in flux control that is dependent on ATP hydrolysis. Since the number of T units in the complexes and their role in detail is yet unknown, questions of how ATP hydrolysis is linked to substrate translocation remain elusive.

As noted at the outset, ECF transporters fall into two subclasses. Whereas members of subclass I like BioMNY have a dedicated A1A2T module, subclass II encompasses promiscuous A1A2T modules that are shared by many diverse S components in one organism. Whether or not members of the two subclasses have the same oligomeric composition and translocate their substrates in the same way, is unknown. It has been suggested that ThiT of the subclass II thiamine transporter may act as a true substrate-capture protein that binds thiamine with picomolar affinity and depends on an A1A2T module for

substrate release into the cytoplasm [5]. This model is not compatible with available data for subclass I systems, namely the biotin transporter [[7] and the present work] and two Co^{2+} transporters [26, 27]. It was shown in the latter three cases that the S units can act as stand-alone importers, albeit they are less efficient in comparison to the holotransporters. Application of fluorescent techniques on living cells in future experiments may help characterize the differences between members of the two subclasses of ECF transporters as regards oligomeric composition.

AUTHOR CONTRIBUTION

Friedrich Finkenwirth, Olivia Neubauer, Julia Gunzenhäuser and Janna Schoknecht performed research; Silvia Scolari, Martin Stöckl and Thomas Korte helped with data analysis and maintenance of equipment; Andreas Herrmann and Thomas Eitinger are the principal investigators, designed the research and wrote the paper.

ACKNOWLEDGEMENTS

We thank Jörg Nikolaus (Institut für Biologie/Molekulare Biophysik, Humboldt-Universität zu Berlin) for assistance with fluorescence microscopy, and Edward Schwartz (Institut für Biologie/Mikrobiologie, Humboldt-Universität zu Berlin) for critical comments on the manuscript.

FUNDING

This work has been funded by the Deutsche Forschungsgemeinschaft [grant numbers Ei 374/3-1, Ei 374/4-1 to T.E., and grant numbers GRK 1121, He 3763/13-1 to A.H].

REFERENCES

- 1 Rodionov, D. A., Hebbeln, P., Eudes, A., ter Beek, J., Rodionova, I. A., Erkens, G. B., Slotboom, D. J., Gelfand, M. S., Osterman, A. L., Hanson, A. D. and Eitinger, T. (2009) A novel class of modular transporters for vitamins in prokaryotes. *J. Bacteriol.* **191**, 42-51
- 2 Eitinger, T., Rodionov, D. A., Grote, M. and Schneider, E. (2010) Canonical and ECF-type ATP-binding cassette importers in prokaryotes: diversity in modular organization and cellular functions. *FEMS Microbiol. Rev.*, doi:10.1111/j.1574-6976.2010.00230.x
- 3 Duurkens, R. H., Tol, M. B., Geertsma, E. R., Permentier, H. P. and Slotboom, D. J. (2007) Flavin binding to the high affinity riboflavin transporter RibU. *J. Biol. Chem.* **282**, 10380-10386
- 4 Eudes, A., Erkens, G. B., Slotboom, D. J., Rodionov, D. A., Naponelli, V. and Hanson, A. D. (2008) Identification of genes encoding the folate- and thiamine-binding membrane proteins in Firmicutes. *J. Bacteriol.* **190**, 7591-7594
- 5 Erkens, G. B. and Slotboom, D. J. (2010) Biochemical characterization of ThiT from *Lactococcus lactis*: a thiamin transporter with picomolar substrate binding affinity. *Biochemistry* **49**, 3203-3212
- 6 Neubauer, O., Alfandega, A., Schoknecht, J., Sternberg, U., Pohlmann, A. and Eitinger, T. (2009) Two essential arginine residues in the T components of energy-coupling factor transporters. *J. Bacteriol.* **191**, 6482-6488
- 7 Hebbeln, P., Rodionov, D. A., Alfandega, A. and Eitinger, T. (2007) Biotin uptake in prokaryotes by solute transporters with an optional ATP-binding cassette-containing module. *Proc. Natl. Acad. Sci. U. S. A.* **104**, 2909-2914
- 8 Day, R. N. and Schaufele, F. (2008) Fluorescent protein tools for studying protein dynamics in living cells: a review. *J. Biomed. Opt.* **13**, 031202
- 9 Levitt, J. A., Matthews, D. R., Ameer-Beg, S. M. and Suhling, K. (2009) Fluorescence lifetime and polarization-resolved imaging in cell biology. *Curr. Opin. Biotechnol.* **20**, 28-36
- 10 Wang, Y., Shyy, J. Y. and Chien, S. (2008) Fluorescence proteins, live-cell imaging, and mechanobiology: seeing is believing. *Annu. Rev. Biomed. Eng.* **10**, 1-38
- 11 Berney, C. and Danuser, G. (2003) FRET or no FRET: a quantitative comparison. *Biophys. J.* **84**, 3992-4010
- 12 Scolari, S., Engel, S., Krebs, N., Plazzo, A. P., De Almeida, R. F., Prieto, M., Veit, M. and Herrmann, A. (2009) Lateral distribution of the transmembrane domain of influenza virus hemagglutinin revealed by time-resolved fluorescence imaging. *J. Biol. Chem.* **284**, 15708-15716
- 13 Rizzo, M. A., Springer, G. H., Granada, B. and Piston, D. W. (2004) An improved cyan fluorescent protein variant useful for FRET. *Nat. Biotechnol.* **22**, 445-449
- 14 Engel, S., Scolari, S., Thaa, B., Krebs, N., Korte, T., Herrmann, A. and Veit, M. (2010) FLIM-FRET and FRAP reveal association of influenza virus haemagglutinin with membrane rafts. *Biochem. J.* **425**, 567-573
- 15 Hadley, A. (2008) CombineZM
[<http://www.hadleyweb.pwp.blueyonder.co.uk/CZM/combinezm.htm>].
- 16 Schattat, M. H. and Klösgen, R. B. (2009) Improvement of plant cell microscope images by use of 'Depth of Field' - extending software. *Endocytobiosis Cell Res.* **19**, 11-19
- 17 Shaner, N. C., Patterson, G. H. and Davidson, M. W. (2007) Advances in fluorescent protein technology. *J. Cell Sci.* **120**, 4247-4260
- 18 Hebbeln, P. and Eitinger, T. (2004) Heterologous production and characterization of bacterial nickel/cobalt permeases. *FEMS Microbiol. Lett.* **230**, 129-135

- 19 Davidson, A. L., Dassa, E., Orelle, C. and Chen, J. (2008) Structure, function, and evolution of bacterial ATP-binding cassette systems. *Microbiol. Mol. Biol. Rev.* **72**, 317-364
- 20 Scheu, P. D., Liao, Y. F., Bauer, J., Kneuper, H., Basche, T., Unden, G. and Erker, W. (2010) Oligomeric sensor kinase DcuS in the membrane of *Escherichia coli* and in proteoliposomes: chemical cross-linking and FRET spectroscopy. *J. Bacteriol.* **192**, 3474-3483
- 21 Slotboom, D. J., Duurkens, R. H., Olieman, K. and Erkens, G. B. (2008) Static light scattering to characterize membrane proteins in detergent solution. *Methods* **46**, 73-82
- 22 Li, H. and Turner, R. J. (2009) In vivo associations of *Escherichia coli* NarJ with a peptide of the first 50 residues of nitrate reductase catalytic subunit NarG. *Can. J. Microbiol.* **55**, 179-188
- 23 Margolin, W. (2000) Green fluorescent protein as a reporter for macromolecular localization in bacterial cells. *Methods* **20**, 62-72
- 24 Vats, P., Yu, J. and Rothfield, L. (2009) The dynamic nature of the bacterial cytoskeleton. *Cell. Mol. Life Sci.* **66**, 3353-3362
- 25 Hoppe, A., Christensen, K. and Swanson, J. A. (2002) Fluorescence resonance energy transfer-based stoichiometry in living cells. *Biophys. J.* **83**, 3652-3664
- 26 Rodionov, D. A., Hebbeln, P., Gelfand, M. S. and Eitinger, T. (2006) Comparative and functional genomic analysis of prokaryotic nickel and cobalt uptake transporters: evidence for a novel group of ATP-binding cassette transporters. *J. Bacteriol.* **188**, 317-327
- 27 Siche, S., Neubauer, O., Hebbeln, P. and Eitinger, T. (2010) Bipartite S unit of an ECF-type cobalt transporter. *Res. Microbiol.*, in press

Figure legends

Figure 1 Localization of mCer-tagged biotin-transport proteins and of the mYFP-tagged NiCoT-type Co^{2+} transporter by confocal fluorescence microscopy

Cell suspensions were treated with the membrane marker FM4-64, mixed with low-melting agarose, and subjected to microscopy. Images were collected sequentially at excitation wavelengths of 458 nm (mCer) and 559 nm (FM4-64) nm for mCer-containing samples, and 515 nm (mYFP) and 559 nm (FM4-64) for mYFP-containing samples. Upper panels, FM4-64 fluorescence was detected between 570 and 670 nm. Lower panels, emission was recorded between 475 and 500 nm for mCer, and between 530 and 545 nm for mYFP.

Figure 2 Stability of fluorescent protein-tagged biotin-transport proteins in extracts of recombinant *E. coli* cells

Cells producing biotin-transport proteins as indicated were harvested in the exponential phase upon growth (3 h) under inducing conditions. The cells were washed and lysed in the presence of SDS-containing sample buffer. Proteins were separated by SDS-PAGE and blotted onto nitrocellulose membranes. The membranes were probed with monoclonal anti-oligo-His (right-hand part) or anti-FLAG antibodies (left-hand part). Proteins (bold letters) and their tags (underlined) detected in each lane are highlighted.

Figure 3 Biochemical properties of fluorescent protein-tagged biotin transporter complexes

(A) Biotin-uptake activity of recombinant *E. coli* S1039 producing fluorescent protein-tagged BioY. Buffered cell suspensions were incubated for 4 hours in the presence of 4 nM [^3H]biotin. Cell-bound radioactivity was determined by liquid scintillation counting upon filtration of 0.5 ml samples through nitrocellulose filters. Non-tagged BioY and BioMN served as positive and negative controls, respectively. Bars represent the mean value of four determinations $\pm$ the standard deviation. (B) Analysis of tagged BioMN and BioMNY complexes by SDS-PAGE/Coomassie staining (a, d, g, j, m, p) and Western blotting with anti-FLAG (b, e, h, k, n, q) and anti-oligo-His antibodies (c, f, i, l, o, r). The protein complexes were solubilized from membranes of recombinant *E. coli* using the non-denaturing detergent dodecyl- β -D-maltoside, and purified by Ni-chelate affinity chromatography. (C) ATPase activity of purified BioMNY transporter variants was determined by quantitating the amount of inorganic phosphate released from ATP. Bars represent the mean value of quadruple determinations, and error bars represent the standard deviation.

Figure 4 FRET analysis of BioM interactions

Lifetime-based FRET spectroscopy with living bacteria was performed as described in Materials and Methods. FRET data for pairs of donor- and acceptor-tagged BioM in the absence (A) and presence (B) of BioNY are shown in the upper part. Panel (C) illustrates FRET for the BioMN–BioM combination with donor-tagged BioN and acceptor-tagged BioM. Asterisks in the heading of A–C mark the fluorescent protein-tagged components. The values in the left panels of A–C (Samples $\pm$ SD) represent fluorescence lifetimes $\tau \pm$ the standard deviation of individual determinations of the donor in the absence (open bars) and presence (black and gray bars) of an acceptor. The middle parts of A–C show the averaged fluorescence lifetimes $\tau \pm$ the standard error of the mean (Means $\pm$ SEM). Results were statistically significant at p values < 0.05 using two-tailed paired t tests. The right-hand panels illustrate the results of individual control experiments with untagged BioM and NiCoT-mYFP replacing BioM-mYFP.

Figure 5 FRET analysis of BioY-BioY interactions

Interactions between BioY peptides in the absence (**A**) and presence (**B**) of BioMN were analyzed by lifetime-based FRET spectroscopy. The figure is organized essentially as described in the legend to Figure 5. The values represent the fluorescence lifetimes τ of individual determinations (left parts in **A** and **B**) and the averages derived thereof (middle parts). Results were statistically significant at p values < 0.05 using two-tailed paired t tests. The right-hand panels show the results of individual control experiments with untagged BioY (**A** and **B**) and NiCoT-mYFP (**A**) replacing BioY-mYFP.

Fig. 1

Fig . 2

Fig . 3

A BioM* - BioM*

B BioM*NY - BioM*

C BioMN* - BioM*

Fig. 4

A BioY* - BioY*

B BioMNY* - BioY*

Fig. 5