ATP-dependent Conformational Changes Trigger Substrate Capture and Release by an ECF-type Biotin Transporter*

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*This work was supported by the Deutsche Forschungsgemeinschaft through Paketantrag PAK459 by Grants STE640/10 (to H. J. S.), SCHN274/49-30-20938102; E-mail: thomas.eitinger@cms.hu-berlin.de.

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The term “energy-coupling factor” (ECF) was coined in the 1970s during experiments on vitamin uptake by lactic acid bacteria. This work demonstrated that the vitamin transport systems consisted of a specific transmembrane binding protein (later called S component) plus an unknown part, the ECF. The latter was required for energization of substrate transport and was shown to be shared by various S components.

About 30 years later, ECF transporters were identified as a large family of prokaryotic uptake systems for vitamins and their precursors and degradation products, for nickel and cobalt ions, for other micronutrients, and for the amino acid tryptophan. The nature of the ECFs was elucidated. They consist of pairs of ATPases with ATP-binding cassettes (ABCs) plus a membrane-bound T component. T components have varying numbers of transmembrane segments and two conserved cytoplasmic helices with a characteristic arginine-containing motif at their C-terminal ends.

ECF transporters were divided into two subgroups based on utilization of a dedicated (group I) or a shared (group II) ECF. General features of ECF transporters are illustrated in the schematic shown in Fig. 1.

Crystal structures of the individually purified S components RibU (for riboflavin), ThiT (for thiamin), BioY (for biotin), and NikM (for Ni²⁺) revealed a surprisingly conserved topology with six membrane-spanning segments in the absence of significant primary structure similarity. Substrate specificity is primarily modulated by variations within transmembrane helices IV–VI among the vitamin-specific S components and by an

JULY 3, 2015 • VOLUME 290 • NUMBER 27
16929
THE JOURNAL OF BIOLOGICAL CHEMISTRY
Here, we attempted to gain experimental insight into reorientations of an ECF transporter during substrate translocation, choosing a subgroup I biotin transporter (BioMNY) as the model. For the majority of experiments, complexes embedded in lipid bilayer nanodiscs were used. We show that (i) binding of ATP induces a reorientation of the BioM<sub>X</sub> ATPase dimer and eventually of the BioY S unit as a requirement for biotin capture, and (ii) subsequent hydrolysis of ATP leads to biotin release.

**Experimental Procedures**

**Bacterial Strains and Plasmids—Escherichia coli** UT5600 containing plasmid pFDX500 (lac<sup>F</sup>) was used as the host for production of BioMNY complexes and solitary BioY variants as described previously (6, 24). Biotin transport activity of BioMNY variants with amino acid replacements was tested with growth assays using a biotin-auxotrophic and intrinsically biotin transport-deficient *E. coli* strain (25, 26). Construction of plasmids pRcBioMNY (6) and pRcBioY (24) encoding the *Rhodo bacter capsulatus* biotin transporter, with an N-terminally deca-His-tagged BioM, a C-terminally c-Myc-tagged BioN, and a C-terminally FLAG-tagged BioY, and the solitary BioY protein with N-terminal His and C-terminal FLAG tags was reported previously.

Plasmids encoding BioMNY variants with single amino acid replacements were constructed by two rounds of PCR. In the first round, a mutagenic forward or reverse primer was used in combination with a primer hybridizing 3’ or 5’ of the nucleotide replacement site. The purified product of this PCR was used as a primer in the second round of PCR together with a primer that anneals at an adjacent site. The amplicon was treated with appropriate restriction endonucleases, and the resulting product was used to replace the corresponding wild-type fragment. For generation of plasmids encoding BioMNY with replacements in BioN and BioY, the individually mutagenized alleles were combined by restriction digestion and ligation. For transfer of *bioY* mutations from pRcBioMNY to pRcBioY, full-length *bioY* was amplified with mutant pRcBioMNY as the template and primers that add recognition sites for Ncol and BglII to the ends, the amplicon was treated with Ncol and BglII, and the product was used to replace wild-type *bioY*. All synthetic DNAs were verified by nucleotide sequencing.

**Purification of Biotin Transporter Variants**—BioMNY complexes and solitary BioY variants were purified as described previously (19, 24). Briefly, recombinant *E. coli* UT5600 cells were grown in a 2-liter culture volume in lysogeny broth. The harvested cells were broken in a French pressure cell, and membranes were pelleted by ultracentrifugation and subsequently solubilized in DDM-containing buffer. The biotin transporter variants were purified via nickel-chelate affinity chromatography, concentrated in Amicon filtration units (Merck-Millipore), passed through PD-10 columns (GE Healthcare), and stored in 50 mM Tris-HCl, pH 7.5, 300 mM NaCl, 15% (v/v) glycerol, 0.05% (w/v) DDM. In the case of BioM<sub>D8SC-NY</sub>, BioM<sub>H177C-NY</sub>, and BioM<sub>Q88C-NY</sub> variants, the recombinant host cells were disrupted in the presence of 5 mM DTT, and all additional N-terminal helix in the metal-binding proteins. Consistent with the previously observed very low dissociation constants for their substrates in the low nanomolar or picomolar range (8, 9, 11–13), the S units were co-crystallized with their substrate in the binding pocket.

X-ray crystallography of the EcfA1A2 ATPase dimer of *T. maritima* (14) and of the *Lactobacillus brevis* ECF holotransporters EcfA1A2T coupled with either FolT (for folates (15)), HmpT/PdxU2 (the substrate specificity of which has not been determined experimentally (16)), or PanT (for pantothenate (17)) provided novel structural insights and led to hypotheses about the transport mechanism. The subunit stoichiometry of ECF transporters has been discussed controversially. Whereas oligomeric forms of S and T units were reported (6, 14, 18–20), the A1/A2/T/S components of *Lactococcus lactis* subgroup II transporters were found predominantly in a 1:1:1 stoichiometry (21). Consistent with the latter, the asymmetric unit in crystals of the three *L. brevis* subgroup II transporters contained one copy each of A1, A2, T, and S. As an unprecedented finding, the S units were oriented almost perpendicularly to the transmembrane segments of the T unit and thus parallel to the membrane. Because the complexes contained neither substrates in the S units nor nucleotides bound to the ATPases, it was concluded that the three structures represent a resting state of the transporters (15–17, 22, 23). A model was proposed according to which the S units undergo a rotation by almost 90° within the membrane (see the schematic shown in Fig. 1) in order to allow substrate binding at the outer surface of the membrane. This model, however, is highly speculative because biochemical evidence is lacking, and the crystal structure of a single state cannot provide information on dynamic rearrangements.

**Mechanism of ECF Transporters**

**FIGURE 1. General features of ECF transporters.** The ECF consists of a membrane-bound T component and two cytoplasmic ABC ATPases (A1 and A2). T components contain 4–5 transmembrane helices in most cases plus three cytoplasmic coupling helices (CHs). Characteristic xRx signatures (where x represents a small residue like Ala, Gly, Ser, or Leu) are located at the C-terminal end of CH2 and CH3. The Arg residues dock into an acidic groove of the ATPases. The red rectangle in T-CH2 indicates a potential contact site with an AXXxA or related signature in TMH1 of the S components (also illustrated as a red rectangle). In crystallized ECF transporters, the brown rectangle in T-CH3 was located in proximity to the corresponding brown segments in TMH1, -2, and -6 of the S components. The orange loop connecting TMH1 and -2 in the S components is predicted to play a central role in locking and opening of the substrate binding pocket. Hypothetically, S components rotate within the membrane during the transport cycle. The beige-colored cylinders in A1 and A2 represent the Q-helices of the ATPases. Q-helices are a typical feature of the ATPases of ECF transporters and contain a Gln residue in a conserved signature of six amino acid residues (14). The Q-helices are predicted to approach each other (indicated by convergent arrows) after ATP binding and closure of the nucleotide-binding sites.
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subsequent purification steps were performed in the presence of 2 mM DTT to avoid spontaneous disulfide formation.

Mass Spectrometry—The amount of biotin bound to BioY or BioMNY complexes upon isolation was quantified by an HPLC-coupled electrospray ionization time-of-flight mass spectrometric assay as described (19).

Preparation of Nanodiscs—Nanodiscs containing wild-type BioMNY or its variants were essentially prepared as described elsewhere (27, 28) using tagless membrane scaffold protein MSP1E3D1 and E. coli total lipids (Avanti Polar Lipids). Lipids (8.3 mg) were dried in a rotating vacuum evaporator and kept in an exsiccator under vacuum overnight. Subsequently, the dried lipid film was resuspended by sonication for 5 min in a bath-type sonicator after addition of 900 μl buffer (20 mM Tris-HCl, pH 7.4, 100 mM NaCl) and 87 μl of a DDM solution (10% in the same buffer). BioMNY complex protein (0.96 mg), membrane scaffold protein (1.62 mg), and the lipid preparation were mixed at a molar ratio of 1.5:1:200, assuming a subunit stoichiometry of BioY/BioN/BioM of 1:1:2. Buffer was added to reduce the glycerol concentration to below 3%, and the mixture was incubated at 4 °C for 1 h. Biobeads (Bio-Rad) (1.5 g) were added, and the reconstitution mixture was incubated at 4 °C for 4 h under gentle shaking. All of these steps were performed under dinitrogen atmosphere. Purification of assembled nanodiscs was performed by metal-chelate affinity chromatography using nickel-nitrilotriacetic acid-agarose (Invitrogen), thereby taking advantage of the N-terminal deca-His tag on the BioM subunits. After washing the resin with buffer in the presence of 25 mM imidazole, protein was eluted with 250 mM imidazole. Protein-containing fractions were pooled, concentrated, and buffered in 50 mM Tris-HCl (pH 7.5) by passage through a desalting column (PD Mini-Trap G-25, GE Healthcare). Protein concentrations within the nanodiscs were estimated by pixel integration wavelength of 340 nm at room temperature. For reference spectra with the fluorophore in a hydrophilic environment, MIANS (2.5 μM) was reacted with DTT (10 μM) in detergent solution or in nanodisc buffer in the absence of protein.

Spin Labeling with MTSSL—Nanodisc-reconstituted BioM947CNY was incubated with 1 mM DTT for 1 h at 4 °C under gentle shaking to reduce spontaneously formed disulfides between the Cys-87 residues of two BioM molecules. DTT was then removed by a Zeba Spin Desalting Column (Pierce). The samples were subsequently incubated with MTSSL at a 20-fold molar excess of the label at 4 °C in 20 mM Tris-HCl, 100 mM NaCl, pH 7.4, under gentle shaking in the dark. Unbound label was removed, and the samples were concentrated using Amicon Ultra 30K centrifugal filter devices (Merck-Millipore). Spin labeling had only a moderate effect on ATPase activity. The spin-labeled sample retained ~80% of ATPase activity.

Sample Preparation for Pulse EPR (DEER) Experiments—Nanodisc-embedded BioM947CNY with a spin concentration of ~40 μM was treated with 2 mM ATP plus 5 mM EDTA or with 4 mM AMP-PNP plus 5 mM MgCl2 to generate the nucleoside triphosphate-bound state of the transporter. The post-hydrolysis state was produced either by the addition of 2 mM ATP plus 2.5 mM MgCl2 and incubation for 10 min at 37 °C or by the addition of 5 mM ADP plus 2.5 mM MgCl2. All samples were supplemented with glycerol (20%, w/v) as a cryoprotectant, transferred into EPR quartz capillaries, and flash-frozen in liquid nitrogen.

Pulse EPR Experiments—DEER experiments were performed at X-band frequencies (9.3–9.4 GHz) with a Bruker Elexys 580 spectrometer equipped with a Bruker Flexline split ring resonator ER 4118X-MS3 and a continuous flow helium cryostat (CF935, Oxford Instruments) controlled by an Oxford Intelligen-temperature Controller (ITC 503S). All measurements were performed using the four-pulse DEER sequence: \[ \pi/2(v_{\text{obs}} - \tau_1 - \pi - \pi(v_{\text{obs}} - t' - \pi(v_{\text{pump}}) - (\tau_1 + \tau_2 - t') - \pi(v_{\text{obs}}) - \tau_2) - \text{echo} (32). \] Time \( t' \) is varied, whereas \( \tau_1 \) and \( \tau_2 \) are kept constant. The dipolar evolution time is given by \( t = t' - \tau_1 \). Data were analyzed only for \( t > 0 \). The resonator was over-coupled, and the pump frequency, \( v_{\text{pump}} \), was set to the center.
of the resonator dip (coinciding with the maximum of the nitroxide EPR spectrum), whereas the observer frequency, $\nu_{\text{obs}}$, was 65 MHz higher (low field local maximum of the spectrum). All measurements were performed at a temperature of 50 K with observer pulse lengths of 16 ns for $\pi$ and 32 ns for $\pi/2$ pulses and a pump pulse length of 12 ns. Proton modulation was averaged by adding traces at eight different $\tau_1$ values, starting at $\tau_{1,0} = 200$ ns and incrementing by $\Delta \tau_1 = 8$ ns. Data points were collected in 8-ns time steps or, if the absence of fractions in the distance distribution below an appropriate threshold was checked experimentally, in 16-ns time steps. The total measurement time for each sample was 24–48 h. Analyses of the data were performed with DEER-Analysis 2011 (33).

**Biotin Capture Assay**—Nanodisc-embedded BioMNY variants (200 pmol of protein assuming a BioM/BioN/BioY stoichiometry of 2:1:1) in a volume of 200 μl were incubated with 1 nmol of [3H]biotin (89 Ci/mol) or with the same amount of biotin plus mixtures of either 2 mM ATP and 0.5 mM EDTA or 2 mM ATP and 2.5 mM MgCl$_2$ for 20 min at 37 °C under gentle shaking. Unbound biotin was removed via gel filtration in PD Mini-trap G-25 desalting columns, and the samples were concentrated to yield a volume of ~125 μl using Amicon units. The radioactivity in 50 μl of the concentrate was quantified by liquid scintillation counting, and the protein content was estimated by SDS-PAGE and staining.

**Results**

**ATPase Activity of BioMNY Reconstituted into Nanodiscs**—The BioMNY complex was embedded in lipid nanodiscs in order to study the transporter’s properties in a more natural environment (34), and ATPase activity was chosen as an indicator of its functional state. Nanodiscs are composed of a phospholipid bilayer domain with two copies of an amphipathic membrane scaffold protein that wrap around the periphery of the bilayer. They form by removal of detergent from initial detergent-phospholipid micelles. When a membrane protein is added, it is trapped within the forming nanodisc structure. The advantage over proteoliposomes lies in the accessibility of the membrane protein from both sides of the bilayer. Because two membrane scaffold protein molecules form a single nanodisc, a membrane scaffold protein/BioM ratio of ~1:1, as inferred by the pixel analysis, indicated that one BioMNY complex (assuming two copies of BioM per complex, which is supported by crystal structures of the *L. brevis* ECF transporters) (15, 16) was present per nanodisc (Fig. 2A).

Nanodisc-embedded BioMNY displayed an ATPase activity of 0.73 ± 0.01 μmol Pi min$^{-1}$ mg$^{-1}$ (Fig. 2B). This value is slightly higher than what was monitored in detergent solution (0.53 ± 0.04 μmol of Pi min$^{-1}$ mg$^{-1}$) (6). Furthermore, kinetic analysis by a Lineweaver-Burk plot (Fig. 2D) revealed a $V_\text{max}$ of 0.76 ± 0.02 μmol Pi min$^{-1}$ mg$^{-1}$ and a $K_m$ of 0.14 ± 0.02 mM. Correspondingly, a turnover number ($k_{\text{cat}}$) of 1.27 s$^{-1}$ was cal-
ATPase activity was inhibited by the ATP analog AMP-PNP, resulting in ~50% inhibition at a concentration of 4 mM in the presence of 2 mM ATP (Fig. 2E). Kinetic analyses at this concentration ratio detected a $V_{\text{max}}$ of 0.51 ± 0.026 μmol P$_i$ min$^{-1}$ mg$^{-1}$ and a $K_m$ of 0.72 ± 0.02 mM, suggesting that AMP-PNP acts as a competitive inhibitor. Furthermore, the addition of orthovanadate (1 mM) caused 44% inhibition of ATPase activity (Fig. 2C). In case of canonical ABC transporters, orthovanadate traps the transporter in a transition-like state after one step of ATP hydrolysis by blocking release of ADP from the nucleotide binding pocket (36–38). Compared with the majority of well-characterized canonical ABC transporters, the BioMNY ABC transporter seems to be less sensitive to the inhibitor despite the overall structural similarity of the nucleotide binding subunits of both families (4, 14–16). The reason for this observation needs to be elucidated. To our knowledge, inhibition by vanadate has not yet been reported for other ECF-type transporters.

Cys-less BioMNY (containing two Cys-to-Ala exchanges and a deletion of the C-terminal Cys residue in BioM and a Cys-to-Ala exchange in BioN) incorporated into nanodiscs displayed a specific ATPase activity of 0.43 ± 0.015 μmol of P$_i$ min$^{-1}$ mg$^{-1}$, corresponding to about 70% of that of the wild type. Notably, this activity was markedly higher than the previously measured activity in detergent solution (0.17 ± 0.03 μmol of P$_i$ min$^{-1}$ mg$^{-1}$) (6). This finding indicates that the overall effects caused by removal of Cys residues on BioMNY function are largely compensated for by a lipid environment. The moderate inhibitory effect of biotin as observed for the wild type was also largely compensated for by a lipid environment. The moderate inhibitory effect of biotin as observed for the wild type was also largely compensated for by a lipid environment. The moderate inhibitory effect of biotin as observed for the wild type was also largely compensated for by a lipid environment.

**Structural Model of the BioMNY Transporter**—To obtain a more detailed view of the interfaces between BioM, BioN, and BioY in the BioMNY complex, a structural model was calculated with the help of the SWISS-MODEL server (39). BioM and BioN were modeled using one (Protein Data Bank code 4HUQ) of the three available structures of EcfA1A2T from *L. brevis* as the template. This EcfA1A2T has been crystallized individually together with a FoIT (15), a HmpT/(PdxU2) (16) and a PanT (17) S unit in the nucleotide-free and substrate-free state.

BioY was modeled on the basis of the *L. lactis* BioY structure (Protein Data Bank code 4DVE). The LBIOY protein had been purified and crystallized in a solitary and substrate-bound state (9). A typical feature of this structure and the structures of the substrate-loaded RibU (7) and ThIT (8) proteins is the location of the loop region connecting TMH1 and TMH2, which is predicted to face the exterior of the membrane and lies as a lid on the substrate-binding pocket (22, 23). In the substrate-free holotransporter structures, the S units FoIT, PdxU, and PanT are toppled over in the membrane, and this loop is orientated toward the cytoplasm. Repositioning of this loop has been observed in response to thiamin binding to LIThIT by several techniques (40). The modeled BioY illustrated in (Fig. 3) shows loop 1 on the biotin-binding pocket formed by the C-terminal half of the protein, which probably does not reflect the conformation in the substrate-free and nucleotide-free state of the BioMNY transporter.

When run in automated mode, SWISS-MODEL as well as I-TASSER, an alternate server for protein modeling (41), chose the same templates as we did manually. The quality of the modeled BioM1, BioM2, BioN, and BioY structures was judged by comparison with the template structures using the DALILITE algorithm (42). The low root mean square deviation values of 0.8 Å (BioM1 versus EcfA1 for 220 of 284 Cα atoms), 0.5 Å (BioM2 versus EcfA2 for 223 of 275 Cα atoms), 0.6 Å (BioN versus EcEF for 204 of 245 Cα atoms), and 0.4 Å (BioY versus LBIioY for 177 of 188 Cα atoms) underscored the significance of the model. The root mean square deviations of the Cα atoms in the individual structure models obtained with I-TASSER compared with the templates were slightly higher. Therefore, we decided to use the models obtained with SWISS-MODEL.

The quality of the models was further assessed with PROCHECK (43), VERIFY3D (44), and ProSA (45). The integrity of the modeled BioM structures was confirmed. Based on the specific physicochemical properties of membrane proteins, the three-dimensional versus one-dimensional scores obtained by VERIFY3D are generally lower compared with those of soluble proteins. ProSA yielded z-scores for the BioM, BioN, and BioY models within the range of scores typically found for proteins of similar sizes, and thus, the quality of the models was approved.

BioY was docked to BioN using the ZDOCK server (46). The resulting BioMNY model was edited with UCSF CHIMERA (47) and is shown in Fig. 3.
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**Functional State of BioMNY Variants with Amino Acid Replacements**—In order to assign biotin transport activity to recombinantly produced candidate biotin transporters, we have recently constructed a biotin-auxotrophic (ΔbioH) and intrinsically biotin transport-deficient (ΔyigM) *E. coli* strain (25, 26). This strain grows on mineral salts medium supplemented with biotin at micromolar concentrations, but it fails to grow on 1 nm biotin unless it produces a recombinant high affinity biotin transporter. In our previous work (25), we identified many solitary BioY proteins that naturally occur in organisms lacking any recognizable EcfAT module as functional biotin transporters. As indicated in Fig. 4A, the solitary *R. capsulatus* BioY did not stimulate growth on 1 nm biotin. In contrast, the recombinant cells containing BioMNY grew under these conditions, suggesting that in this case, only the tripartite system represents a functional biotin transporter. We used this knowledge to test all BioMNY variants with amino acid replacements constructed in the present study by growth assays. Fig. 4B shows that the majority of exchanges did not interfere with the functional state of the tripartite complexes. Exceptions are the A12W and A13W replacements in BioY (see below) and the E161Q replacement in the BioM ATPase. The latter reduced ATPase activity to background levels, and all three replacements abolished biotin uptake activity. The E161Q exchange removed the so-called catalytic carboxylate immediately adjacent to the Walker B motif. It was previously shown for ATPases of canonical ABC transporters that the conserved glutamate residue at this position is essential for activity (48–50).

**Closure and Reopening of the BioM Dimer**—The nucleotide-binding domains of canonical ABC importers undergo a closure/reopening cycle during the transport process (4, 48, 51). In particular, the distance of the Q-loops in the MalK dimer of the maltose ABC importer MalFGK was demonstrated to decrease in the ATP-bound state and to increase upon ATP hydrolysis (50, 52). A similar response was observed for the HisP dimer of the transporter for positively charged amino acids (HisQMP) (53). In order to test whether closure and reopening occurs in a similar way in ATPases of ECF transporters, we measured distances between the Q-helices of the BioM dimer that correspond to the Q-loops of canonical ABC importers (14). These analyses were performed with nanodisc-embedded monocysteine BioMNY variants harboring BioM₁₂₆₆C, BioM₁₉₇₇C, or BioM₈₈₈₈C replacements. Cross-linking experiments with the D₈₆C and Q₈₈C variants using EBS (5 Å) and HBS (10 Å) in the absence and presence of ATP identified distance changes between the 86/86’ and 88/88’ positions. Whereas ATP resulted in a slight increase in the amounts of EBS-linked product in the case of BioM₄₃₆₆C (1.7-fold by pixel analysis), a substantial increase was observed for EBS-linked (2.6-fold) and HBS-linked products (3.4-fold) in the case of BioM₈₈₈₈C (Fig. 5). These findings are compatible with the notion that the Q-helices approach each other as a consequence of nucleoside triphosphate binding.

A more detailed view was obtained by interspin distance measurements between pairs of nitroxide spin labels at positions 87/87’ in complexes with the BioM₁₉₇₇C variant using DEER spectroscopy. Rotamer analyses (see Ref. 54 for the method and Ref. 55 for a review) predicted various sterically viable orientations of the spin label side chain, and thus, the impact of spin labeling on the protein structure at this position can be assumed to be minor. Fig. 6 summarizes the DEER analyses of samples in the apo-state, in the nucleoside triphosphate-bound state (generated by the addition of ATP or Mg²⁺ plus AMP-PNP), and the post-hydrolysis state (obtained after catalytic turnover of ATP in the presence of Mg²⁺ or by the addition of Mg²⁺ plus ADP). The DEER raw data V(t) (Fig. 6A) reveal a strongly damped oscillation due to spin pair interaction and an additional decay due to a homogenous multidimensional distribution of spins in the samples, the so-called background B(t). Dividing V(t) by B(t) yields the form factors, which are normalized at zero time (Fig. 6B). The modulation depth of 8–12% according to a labeling efficiency of 0.35–0.5 spins/monomer indicates limited accessibility of the 87/87’ positions for MTSSL. Gaussian distance distributions were fit to the form factors F(t). The resulting distance distributions P(d) revealed significant shifts of the maximum populations for each state (Fig. 6C). The distance distribution for the apo-state showed its maximum at 3.4 nm. In the nucleoside triphosphate-bound state (in the presence of ATP or AMP-PNP), this distance was reduced to 2.7 nm. The maxima of both post-hydrolysis samples were shifted to 3.9 nm. Taken together, the pulse EPR

![FIGURE 4](image-url)

_**FIGURE 4. Growth of a biotin-auxotrophic and intrinsically biotin uptake-deficient E. coli strain producing BioMNY variants in vitamin-free mineral salts medium.** A, growth of the strain containing *R. capsulatus* BioMNY (solid) or the derived solitary BioY (hatched). Growth on 1 nm biotin depended on the tripartite system. B, growth of recombinants producing BioMNY variants containing single or double replacements or lacking Cys residues on 1 nm biotin. The values represent the means of double or triple determinations ± S.D. (error bars). D86C, H87C, Q88C, and E161Q are variants of BioM; V143C and V147C are mutants of BioN, and A112W, A112V, A113W, A13V, V16W, A17W, L26C, K137C, and A152C are mutants of BioY._
experiments provided evidence for conformational transitions in the BioM dimer that bring the side chains of positions 87/87’ closely together upon ATP binding and open the interface of the dimer upon ATP hydrolysis.

ATP-induced Rearrangements at the BioN-BioY Interface—Available structural and functional data on subgroup II ECF transporters (8, 17) suggest that an AXXXA motif within the first TMH of the S units interacts with the coupling helix 2 (CH2) of the T units. We analyzed by growth assays the significance of a corresponding A12A13XXV16A17 signature in BioY for activity of the BioMNY transporter upon amino acid replacements. As shown in Fig. 4, bulky tryptophan residues at positions 12 and 13 completely abolished activity, whereas a less dramatic effect for the Trp replacement at position 16 and no effect at position 17 was observed. The results are compatible with the notion that small hydrophobic amino acid residues in the central part of TMH1 of BioY are involved in subunit interactions. Consistent with this idea, replacement of Ala-12 and Ala-13 by valine residues had only little impact on biotin uptake activity (Fig. 4). These results and the spatial proximity of the corresponding segment of BioY to the CH2 of BioN in the BioMNY structure model (Fig. 3) prompted us to investigate BioN-BioY interactions by site-specific cross-linking.

BioMNY variants with a Cys residue in BioN at position 143 or 147 within the CH2 plus a Cys residue in BioY at position 12, 13, 16, or 17 were constructed and cross-linked with the homobifunctional thiol linkers EBS, HBS, and PBS in detergent solution. Among seven combinations tested, significant amounts of cross-linked BioN-BioY were observed for the variants containing the BioNV143C/BioYV16C, BioNV143C/BioYA17C, and BioNV147C/BioYA17C pairs (Fig. 7). In addition to BioN-BioY, BioN dimers were found under most conditions even in the absence of a cross-linker. This observation suggests that BioN dimers arise spontaneously by disulfide bond formation. Oxidation of BioN variants with single Cys residues at the C-terminal side of CH2 and CH3 to form homodimers was observed before (6), but the significance of this observation remains to be clarified. The reactivity of the mono-Cys BioN/mono-Cys BioY pairs with the three cross-linkers differed and was affected by ATP. In the absence of ATP, the BioNV143C/BioYA17C pair was only weakly cross-linked by EBS (5 Å) with some product formed by HBS (10 Å). The pattern changed when ATP was present. Under those conditions, EBS, HBS, and PBS (25 Å) resulted in comparable amounts of product. This finding suggests that binding of ATP to the BioM dimer induces a conformational shift that increases the flexibility of the BioN/BioY interface.
A similar response was observed for the BioN\textsubscript{V143C}/BioY\textsubscript{V16C} pair. The gradual reactivity with EBS/HBS in the absence of ATP was no longer observed in its presence. Under those conditions, all three cross-linkers formed comparable amounts of BioN-BioY heterodimers. In agreement with these results, reorientation of position 147 of BioN in response to the presence of ATP was also observed by fluorescence emission analyses of MIANS-labeled BioMNY (see Fig. 9).

The BioN\textsubscript{V143C}/BioY\textsubscript{V16C} pair was not linked by PBS under any condition. The EBS/HBS reactivity in the absence of ATP was significantly switched to an EBS/HBS reactivity upon its addition (Fig. 7), indicating a shorter distance of the two positions in the presence of nucleotide. These observations demonstrate that charging of the BioMNY complex with ATP alters the geometry of the BioN CH2/BioY-TMH1 interaction site rather than disrupting the domain interactions.

Reorientation of BioY Loop Regions—The hypothetical toppling over mechanism (16, 22, 23) predicts that the 5 units of ECF transporters adopt an upright topology after nucleotide binding to the ATPase dimer. As a result of the ATP-induced rotation, the substrate-binding site would face the extracellular side of the membrane. To test this possibility, we analyzed solvent accessibility to residues within the loop regions of BioY connecting TMH1 with TMH2 and TMH5 with TMH6. These loops are predicted to face the extracellular space in the ATP-bound state of the transporter. Mono-Cys variants of BioY were constructed that contain individual Cys residues in the TMH1/TMH2-connecting (L26C) and TMH5/TMH6-connecting (K137C/A152C) loop. The purified solitary BioY and BioMNY variants were labeled with the environmentally sensitive and thiol-reactive fluorophore MIANS in detergent solution. Its emission maximum responds to the polarity of the environment and is red-shifted by a polar and blue-shifted by a hydrophobic surrounding (56). The labeled probes were analyzed by fluorescence spectroscopy, and the results are shown in Fig. 8.

The blue-shifted fluorescence emission, 3 nm in the case of BioY\textsubscript{L26C} and 6 nm in the case of BioY\textsubscript{K137C/A152C} indicates that the two loops of BioY are trapped in a more hydrophobic environment in the presence of BioMN. A comparable hypsochromic effect was not observed for the BioY\textsubscript{A152C} variant; therefore, this position was not considered further. The shifted fluorescence emissions of MIANS-labeled BioY loops in the presence of BioMNY correlate with significant differences in the biotin content of wild-type solitary BioY and BioMNY complexes in the “as isolated” state. Analysis of the biotin content by a recently developed mass spectrometric assay (19) identified only background levels of biotin in BioMNY but 0.5 mol of biotin/mol of the solitary BioY (not shown). The latter value corresponds to that reported previously (19). This difference suggests that the substrate-binding site of BioY is hardly accessible in BioMNY complexes in the resting state.
Next, we investigated whether the addition of ATP and ATP plus biotin to the BioMNYL26C and BioMNYK137C variants causes a red-shifted emission, indicating exposure to a more polar environment. The MIANS-labeled samples were analyzed in detergent solution as well as in the nanodisc-embedded state (Fig. 9). Indeed, a bathochromic effect was observed in the presence of nucleotide and substrate, suggesting that under these conditions, BioY adopts an upright orientation in the BioMNY complexes that resembles its orientation within the membrane in the solute state.

An opposite response of the fluorescence emission to ATP plus biotin was observed for the MIANS-labeled BioMNYV147CY (Fig. 9). The blue shift by 6 nm in detergent solution and 9 nm in nanodiscs suggests that the CH2 of BioN gets in closer contact to the lipid phase of the membrane as a consequence of ATP binding to BioM.

Biotin Capture and Release by BioMNY Complexes—The above results gave rise to the notion that the observed ATP-induced conformational changes in BioMNY might eventually result in binding of biotin. To address this question, we investigated binding of [3H]biotin to nanodisc-embedded BioMNY variants in the apo-state and in the presence of ligands. The addition of a mixture of ATP and EDTA resulted in a significant biotin capture by the wild-type, Cys-less, and BioME161QNY complexes (Fig. 10). EDTA was added to observe the consequences of ATP binding in the absence of ATP hydrolysis because the latter depends on divalent cations. Removal of its Cys residues did not markedly alter substrate binding to the BioMNY complex.

In a second set of experiments, substrate capture was analyzed in the presence of Mg-ATP. Under these conditions, very small amounts of biotin were captured by wild-type and Cys-less BioMNY, whereas the BioME161QNY variant accumulated biotin as in the presence of ATP/EDTA (Fig. 10). These findings allow the conclusion that ATP hydrolysis releases biotin that was previously captured after ATP binding. Substrate release is blocked by the E161Q replacement in BioM that interferes with ATP hydrolysis.

Discussion

In the crystals of isolated S units, channel-like structures were not observed; hence, the substrate-translocation mechanism of ECF transporters remained enigmatic. The original proposal for RibU that energy input by Ecfa1A2T may transiently cause an inward-open conformation by moving transmembrane helices I–II and IV–VI away from each other (7) was considered unlikely after the molecular dynamics simulation (57). Likewise, the structures of ThIT and BioY did not point to substrate translocation through the inside of S units (8, 9). An alternate picture emerged when the crystal structures of three ECF holotransporters from L. brevis were reported (15–17). Because substrates were not bound to the S components and the ATPase dimers were free of nucleotides, it was proposed that all three structures may represent a postcatalytic state after ATP hydrolysis and substrate release. Intriguingly, the S components were oriented almost parallel to the membrane, and the substrate binding pockets were open and positioned next to the cytoplasmic part of the T component.

This architecture offered a novel possibility of how substrate translocation may be achieved. In contrast to other ABC transporters in which the two transmembrane domains are homodimers or pseudosymmetric heterodimers, T and S components are asymmetric (reviewed in Refs. 22 and 51). Among subgroup II ECF transporters, the same Ecfa1A2T module interacts with various S components with specificity for structurally unrelated compounds. Thus, it is unlikely that substrate passage occurs at the interface between S and T subunits unless a single T component would contribute to the translocation of chemically unrelated molecules. Based on the holotransporter structures, a hypothesis was generated according to which the S components rotate within the membrane, which would allow substrate binding at the outside in the uplifted state and substrate release into the cytoplasm after rotation and opening of the binding pocket. This model resembles the early view of membrane transporters as mobile carriers (58), but experimental evidence for this mechanism by ECF transporters is elusive.

In the present study, we attempted to shed light on the function of ECF transporters by collecting evidence in favor of or against a rotation mechanism, and we chose a subgroup I biotin transporter for investigation. As the basis for further biochemical and biophysical analyses, its stability and ATPase activity in various environments were tested. Reconstitution of BioMNY into proteoliposomes was inefficient, but when embedded in lipid bilayer nanodiscs, the complexes were stable for days without loss of ATPase activity. Therefore, for the majority of analyses, this state of the transporter was used. ATPase activity was competitively inhibited by AMP-PNP but, compared with that of canonical ABC importers, was only moderately sensitive to the well known inhibitor vanadate. Amazingly and in contrast to canonical ABC importers (35), substrate (biotin) did not stimulate ATPase activity. Because mass spectrometric analysis failed to detect biotin in purified wild-type BioMNY, these complexes seem to hydrolyze ATP constitutively in the absence of any biotin. Whether or not such futile ATP consumption occurs in living cells remains to be elucidated.
The focus of the next series of experiments was on potential rearrangements within the BioM$_2$ ATPase dimer as a consequence of ATP binding and subsequent ATP hydrolysis. ABC ATPases are known to undergo a closure of the two nucleotide-binding sites upon ATP binding, each formed by the Walker A region of one monomer and the LSGGQ signature region of the second monomer (reviewed in Refs. 4 and 35). Inspection of the modeled BioMNY structure (Fig. 3) suggested that domain closure would reduce the distance between the Q-helices of the two BioM monomers. The Q-helix was identified in the crystal structure of the *T. maritima* EcfA1A2 dimer. It is formed by six residues (X$\phi$X$\phi$X$\phi$, where$\phi$ represents a hydrophobic residue) and was shown by sequence alignments to be specific for the ATPases of ECF transporters (14). For intersubunit distance analyses by cross-linking and EPR, monocysteine variants of BioM were constructed by individually replacing Asp-86, His-87, and Gln-88 within the Q-helix region. The D86C variant was previously shown to efficiently dimerize by disulfide bond formation (6), suggesting spatial proximity of the 86/86$\phi$ positions. In contrast to observations with corresponding replacements in the *S. thermophilus* EcfA1A2, which strongly affected riboflavin uptake by the EcfA1A2T-RibU transporter (14), those replacements left the biotin uptake function of BioMNY almost unaffected (Fig. 4). Cross-linking in particular of the BioM88/88$\phi$ residues by the 5 Å (EBS) and 10 Å (HBS) linkers was much more pronounced in the presence of ATP, which is compatible with domain closure. The fact that some cross-linking was also observed with the 25-Å linker PBS (not shown) indicated significant flexibility of the Q-helix regions. The cross-linking results were corroborated by interspin distance analyses. For those experiments, position 87 was chosen because rotamer analyses predicted a variety of sterically possible spin label side chain orientations. The calculated C$^\mu\beta$ distances in the model between positions 87/87$'$ are 13 Å. The
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FIGURE 10. [3H]biotin capture of nanodisc-incorporated BioMNY in response to ATP binding and hydrolysis. Protein-containing nanodiscs (wild-type BioMNY (solid), Cys-less BioMNY (open), and BioM2E161QNY (hatched)) were incubated with [3H]biotin, [3H]biotin plus ATP/EDTA, and [3H]biotin plus Mg2+ -ATP. The amount of biotin bound to the protein complexes was quantified by liquid scintillation counting. In the absence of ATP (left bar group), 0.09–0.14, 0.30–0.34, and 0.22–0.32 mol of biotin/mol of BioMNY was identified for the wild-type, Cys-less, and BioM2E161QNY complexes, respectively. The averaged values are defined as 1-fold. Bars with error bars, mean changes of the biotin content (mol of biotin/mol of BioMNY) ± S.D. relative to the ATP-free values in response to treatment with ATP/EDTA and Mg2+ -ATP. The values represent the means of double or triple determinations ± S.D. The observed differences are statistically significant (p < 0.05 in paired two-tailed t tests).

distance between Cγ and the NO group of the spin label side chain is 4–8 Å (59, 60), yielding a maximal theoretical interspin distance of 29 Å. This value is in reasonable agreement with the distances of 34, 27, and 39 Å for the nucleotide-free, ATP-bound, and post-hydrolytic states, respectively, as observed by DEER analyses (Fig. 6). It must be considered in this context that the BioM2 homodimer structure was modeled using the L. brevis EcfA1A2 heterodimer in the EcfA1A2T-FolT complex as the template, a fact that may add some uncertainties to details in the model. In agreement with the cross-linking results, the DEER data revealed significant flexibility of the FQ-helix region and evidenced domain closing and reopening. Interestingly, the EPR data reflect three distinguishable conformations related to the three states of the hydrolysis cycle. Three different cycle-related conformations were also reported for BioN, as predicted by the structure model of BioMNY and as confirmed by the crystal structures of the subgroup II transporters (15–17). The S units are not in distinct contact with the ATPases but are selectively bound by the T unit (15–17). The molecular details of this interaction are not fully understood, but CH2 and CH3 in the T components play a central role. S components contain a structurally conserved surface groove formed by hydrophobic or apolar residues into which CH3 of the T component is embedded (17).

Mutational analysis of ThiI has pointed to the important role of an AXXXA motif in TMH1 for complex stability and function of the thiamin transporter (8). This motif or variants thereof in which Ala residues are predominantly replaced by Val are found in many S components. It resembles the previously identified GXXXG signature, a widespread interaction motif in transmembrane helices (68). The significance of individual small residues in TMH1 for interaction with the T component seems to differ among S components. Whereas the A15W and A19W replacements abolished the function of the thiamin transporter (8), the corresponding exchanges had a less dramatic (A13W) or no detectable consequence (A17W) in the case of the pantothenate transporter (17). Results in the present study on BioY are in agreement with both of these findings. Bulky residues at positions 12 and 13 (A12W and A13W) abolished biotin transport, whereas replacement by Val (A12V and A13V) is tolerated. On the other hand, a Trp residue at position 16 (V16W) had only a moderate effect and almost no effect at position 17 (A17W) (Fig. 4).

The cross-linking data shown in Fig. 7 clearly confirm the spatial proximity of positions 16 and 17 of BioY with the CH2 of BioN, as predicted by the structure model of BioMNY and as seen in the crystal structures of the subgroup II transporters. This indicates a similar organization among subgroup I and II ECF transporters. Cross-linking between BioY and BioN in detergent solution was observed in the presence and absence of ATP for wild-type BioMNY (Fig. 7) and for the BioM2E161QNY variant (not shown) in which the ATP-bound state is stabilized (48–50). Thus, the S component seems to be permanently complexed by the T unit during the transport cycle. Whether this is a specific feature of subgroup I transporters and may be different from subgroup II systems in which S components are exchanged is unknown.

Whether or not substrate capture by BioMNY was the consequence of ATP-dependent rearrangements within the complex and an uplift of BioY was a central question. The results of fluorescence spectroscopy, which showed a more hydrophobic environment of CH2 of BioN and a more hydrophilic surrounding of the loops between TMH1 and -2 and TMH5 and -6 in BioY upon the addition of ATP (Fig. 9), are compatible with the uplift hypothesis. These findings, together with our results on ATP-dependent biotin capture, which is reversed by ATP hydrolysis (Fig. 10), strongly support the model shown in Fig. 11. Similar models were previously proposed by others (15, 16, 22,
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FIGURE 11. Model for BioMNY-catalyzed biotin transport. Binding of ATP (1) to the transporter in its resting state leads to an uplift of BioY without interrupting the physical contact to BioN. Access of biotin to the substrate-binding site in BioY (2) occurs in the ATP-bound state. ATP hydrolysis (3) leads to release of biotin into the cytoplasm; the nucleotide-free system returns into the resting state.

23), but step 1 (i.e. ATP-dependent uplift) and step 2 (i.e. ATP-dependent substrate capture) are now experimentally verified. Step 3 (i.e. substrate release into the cytoplasm) remains hypothetical because our observation that ATP hydrolysis interferes with substrate capture does not formally distinguish between substrate release to the inside or outside. Furthermore, mechanistic variations are likely to exist. In the case of the metal-specific ECF transporters, for instance, the S units contain an extra transmembrane helix at the N terminus. A stretch of nine extra-cytoplasmic amino acid residues, including Met-1 and His-2, is strongly conserved. This segment docks into the binding pocket, and the two N-terminal residues provide three of the four metal ion ligands in the binding site (10, 69). Those S units are accompanied by one or two small transmembrane proteins, which are essential for function and may assist in loading of metal ions into the binding pocket or pulling out the N-terminal segment in order to open the pocket for substrate release (69, 70).

Many questions about ECF transporters, including how S units are exchanged among the subgroup II systems or how the transport function of a number of solitary BioY proteins in the absence of other ECF components (25) is achieved, remain to be answered. The biochemical and biophysical analyses in this study have provided experimental insight into distinct steps of the transport cycle of this fascinating group of membrane transporters.

Acknowledgments—We thank Tuya Enver Assafa and Enrica Bordignon (Freie Universität Berlin) for help with the determination of spin labeling efficiency.

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