

Mechanisms of Biotin Transport

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Abstract

Biotin is an important micronutrient widely employed as an enzyme cofactor in all living organisms, therefore, cells that cannot synthesize biotin *de novo* must import it from the external environment. However, most cells have evolved a specific transport protein to facilitate biotin entry into cells, even if they have the necessary biosynthetic pathways, as it is more energetically efficient to scavenge biotin from the environment. The best-characterized examples of biotin transporters now belong to the bacterial energy coupling factor (ECF) family of vitamin transporters that employ similar but distinct mechanisms of solute uptake to the well studied ABC transporters. Here we review recent studies that shed new light on the structure and function of these important proteins. Studies on biotin transporters from organisms outside the bacterial kingdom are also presented, such as the analogous proteins from yeast, mammals and plants. However, there is a paucity of new information here compared to the ECF examples. Possible applications for exploiting biotin transporters for drug delivery are also examined.

Keywords: Biotin; Import; Energy-coupling factors; ABC transporters; Sodium-dependent multi vitamin transporter

Abbreviations: ABC: ATP Binding Cassette; ECF: Energy Coupling Factor; MCT: Mammalian monocarboxylate Transporter; NBD: Nucleotide Binding Domain; SBP: Solute-Binding Protein; SMVT: Sodium-dependent Multi Vitamin Transporter; TMD: Trans-Membrane Domain; VHT: Vitamin H Transporter

Biotin

The micronutrient biotin (also known as vitamin H or B7) is a small 244 Da watersoluble vitamin essential for life [1-3]. Biotin has a well-defined biological function in organisms from all three domains of life as a cofactor for biotin-dependent enzymes. These enzymes often catalyze key reactions in important metabolic pathways such as gluconeogenesis, amino acid metabolism and fatty acid synthesis [4-6]. For enzymatic activity, biotin must be covalently attached to a conserved lysine residue present in the active site of the biotin-dependent enzyme [2,7,8]. The importance of biotin transporters for living organisms is exemplified from several perspectives.

Mammals cannot synthesise biotin *de novo* but efficiently accumulate biotin from their environment. This is evidenced by congenital abnormality in humans where defects in biotin transport results in biotin dependency [9]. Severe deficiency of biotin leads to various clinical abnormalities such as growth retardation, neurological disorders and skin problems in human patients [5,10,11]. Secondly, although many bacteria, Archaea and plants are capable of both importing and synthesizing biotin, uptake of the vitamin from the environment is energetically more favourable [12]. It requires at least 6 enzymes and 7 ATP equivalents to synthesise one biotin molecule [13]. Hence, having an efficient mechanism to scavenge micronutrients may provide an advantage to these organisms especially at important stages of their life cycles, such as during pathogenesis. The transport of biotin into cells is the subject of this review. Here we have reviewed the literature on the recently characterised energy coupling factor (ECF) proteins – a family of vitamin transporters in bacteria that includes the biotin transporter. Studies into the structure and function of the ABC transporters greatly enhance our knowledge about the mechanism of solute movement in biotin transport. Biotin transporters from other organisms will also be briefly discussed, although these proteins have not been as well characterised as the bacterial ECF transporters.

ABC and ECF Transporters

ABC transporter

The ATP binding cassette (ABC) transporters are a large family of membrane proteins that couple metabolic energy produced from ATP hydrolysis to the movement of substrates across cellular membranes [14]. ABC transporters are classified as importers (those that mediate inward translocation of substrates into the cell) or exporters (those that facilitate extrusion of solutes from the cell). The former are predominantly present in prokaryotes [15-17], whereas the latter are found in both prokaryotes and eukaryotes [18]. ABC importers can be grouped into 2 further subtypes based on their topology in the membrane [19,20]. Type I importers contain 10-14 trans-membrane helices as exemplified by the MalFGK2 protein that has been extensively studied using biochemical, biophysical and structural approaches [21]. Type II importers have up to 20 trans-membrane helices, allowing them to transport substrates that are typically larger than those that use Type I importers [22]. The *Escherichia coli* vitamin B12 importer BtuCDF and its homologue HI470 from *Haemophilus influenzae* are examples of type-II importers whose X-ray crystal structures have been reported [23-25].

ECF transporter

The energy coupling factor (ECF) transporters represent the smallest subclass of ATP-dependent importers that have recently been identified in Archaea and Eubacteria [26,27]. These subclasses of integral membrane proteins are the primary facilitators for trafficking certain vitamins into microorganisms that may or may not possess the enzymes necessary for *de novo* vitamin biosynthesis [26,28]. All

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ECF transporters share three common subunits: the solute-binding subunit (EcfS), soluble nucleotide-binding subunits required for ATP hydrolysis (EcfA) and a membrane embedded permease (EcfT) that helps assemble the complex (Figure 1). The structures of the ECF transporters most closely resemble the Type I ABC transporters described above. ECF transporters are grouped into two classes based on their energising modules (Figure 1). Class I transporters employ a multisubunit ECF module dedicated for a single EcfS subunit [26]. The biotin transporter BioMNY from *Rhodobacter capsulatus* is the most well-characterised example of this class [29]. Class II ECF transporters utilise an ECF module encoded by *ecfA*, *ecfA'*, and *ecfT* genes that is shared by multiple EcfS components and, therefore, actively imports a broad range of chemically distinct vitamins [26,30]. While Class I ECFs are predominant in Proteobacteria and Actinobacteria, Class II are prevalent in Firmicutes [26]. Genes encoding the EcfS, EcfT and EcfA subunits are co-located in an operon in the majority of Class I ECF transporters. In contrast, the genes for the energising module of Class II ECF transporters are present in an operon but separated from the genes encoding the EcfS subunit [28,29]. Certain micro-organisms employ both Class I and Class II ECF transporters for importing different substrates [27]. Co-occurrence of the two classes in these Archaea and bacteria [28,29] is an unexpected finding.

Molecular Organisation

Extensive biochemical, biophysical and structural data from studies on the ABC transporters have provided a great deal of insight into the structure and function of these proteins (reviewed [31,32]). In contrast the ECF transporters, which have been identified relatively recently, have not been as well characterised [27,33]. The available data indicate that the ABC and ECF transporters share a number of common but distinct structural and functional features. Both contain a binding protein for capturing substrate, a means for coupling ATP hydrolysis to substrate translocation, and a pathway for transferring substrates across the lipid bilayer [19]. In the well-characterised (classical or canonical) ABC transporters these entities are represented by four or more individual subunits encoded by separate genes [31,32]. The core components consist of a pair of hydrophobic trans-membrane domains (TMDs) that create the trans-membrane tunnel and two soluble ATPase subunits that couple the hydrolysis of ATP to the transport of solutes [18]. An additional periplasmic component, known as the solute or substrate binding protein (SBP), only found

in ABC importers, determines substrate specificity by recognising an appropriate substrate and delivering it to the translocation pore [34]. The molecular organisation of ABC importers in comparison to those of ECF transporters are depicted in Figure 1. The ECF transporters are functionally analogous to ABC importers but with several notable differences. Firstly, the ECF transporters do not possess a SBP that brings ligand to the translocation pore. Instead, a membrane integrated, substrate-specific protein (the EcfS component) is employed to capture and translocate solutes into cells [26,35]. Secondly, ECF transporters utilise an energising module (ECF complex) that consists of a membrane-bound permease in complex with soluble ATPases that reside in the cytoplasm. The stoichiometry of the functional ECF transporter complex still requires elucidation. It has been proposed that the complex assembles with a 1 EcfA:1 EcfA';:1 EcfT:1 S subunit architecture [26,35-37]. This is supported by data from size exclusion chromatography multi-angle light scattering (SEC-MALS) of purified complexes and seen in the currently published X-ray crystal structures of folate and hydroxymethyl pyrimidine transporter complexes reconstituted from purified subunits in proteoliposomes [36,38-40]. Other architectures in which one or more EcfS components assemble with the ECF module have also been proposed [27,41]. Evidence for ECF transporters having more than one EcfS or EcfT subunits have come from *in vivo* FRET assays using fluorescently labelled ECF transporters [42]. These different models might be influenced by two factors. First, ECF transporters naturally have dynamic stoichiometry, allowing them to have one or more Ecf components assembled to the ECF module. Alternatively, *in vitro* reconstitution might not faithfully replicate the natural assembly of the native protein complex [36]. The subunit stoichiometry of ECF transporters may influence transporter activity. This was observed in an *in vivo* study the biotin transporter from *R. capsulatus*, BioMNY. Import studies using radiolabelled biotin were performed with *E. coli* expressing recombinant BioY (ie the EcfS subunit specific for biotin) either alone or in combination with ATPase BioM and permease BioN [26]. Surprisingly *E. coli* cells expressing BioY alone were able to mediate substrate transport. Without the dedicated BioMN energising module, BioY is a low affinity transporter capable of transporting 100-400 nM ³H-biotin [26,29]. This is in contrast to the high affinity complex containing BioY, BioM and BioN that performs efficient biotin translocation in low (<5 nM) ³H-biotin concentrations [29]. The biotin transporter from *R. capsulatus* is the only prokaryotic system that has so far been demonstrated to function in low or high affinity states [26,29]. Other vitamin transporters such as ThiT (thiamin), RibU (riboflavin), and HmP (hydroxymethyl pyrimidine) require preassembly with the ECF module for activity [27,36,37]. Without the ECF energising module, these EcfS components can bind substrates but are unable to transfer them across biological membranes [36,37]. Recombinant BioY proteins from proteobacteria that naturally lack an ECF module can only facilitate low affinity biotin import into *E. coli* K12 [36,37].

Structural Biology of the Functional Subunits

In this section we review the literature on the known structures of the individual subunits of the ECF transporters with a view to describing the assembly of the transporter complexes and how this relates to the function of the transporters.

Nucleotide-binding domain

The nucleotide-binding domains (NBDs) in the ATPase subunits are hallmarks of ABC transporters due to their high amino acid

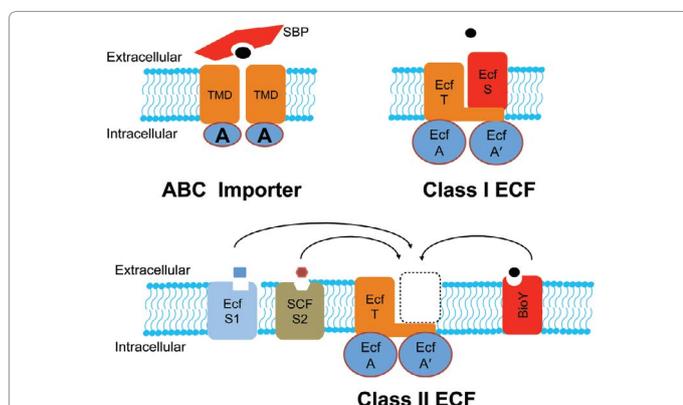


Figure 1: Comparison of subunit architectures of an ABC importer and ECF transporters.

For Class 1 transporters the energy-coupling factor is designated for a single substrate component, whereas this is shared with different EcfS subunits in Class II transporters.

sequence conservation and their occurrence in all ATP-dependent transporters [19]. Conserved structural motifs that are important for NBD function include the Walker A (also known as P-loop) and B motifs, family signature region (also known as H-loop or C switch motif), A-, D-, and Q-loops, and a short LSGGG motif [20,43]. Available crystal structures of isolated NBD monomers and dimers show that these motifs are segregated into an ATPase subdomain and an α -helical subdomain connected by two flexible loops [14,42]. All motifs found in the NBD subunits of classical ABC transporters are also present in each ATPase subunit of the ECF transporters (Figure 2), implying a high degree of functional conservation [29]. Indeed, BioM, EcfA or EcfA', of *Thermotoga maritima* show high (60%) amino acid sequence similarity to the NBD subunits of the classical ABC transporters [27]. Conserved motifs in the ATPase of ECF transporters are segregated into three subdomains: Rec-A like subdomain, helical subdomain and C-terminal subdomain [27,39]. The Rec-A like subdomain is an ATP binding site containing the Walker A and Walker B motifs [27,39]. The helical subdomain consists of a three-helix bundle with the LSGGQ signature critical for nucleotide binding. The C-terminal subdomain is a ~40-residue region that encompasses all regions of the helical subdomain [27].

Trans-membrane domain (TMD)

The TMDs of ABC transporters are found as either homo- or heterodimers [19,44]. In contrast, the trans-membrane component of ECF transporters always consists of two distinct proteins, namely the EcfT and EcfS. The permease EcfT shows low sequence similarity to the trans-membrane subunits of the classical ABC transporters [45]. Between species the sequence identity for the EcfT is <20% [39]. This low sequence identity is likely to contribute to the specific assembly of functional complexes required for certain vitamins. EcfT is thought to be responsible for stabilising the interaction between the EcfS component and the soluble ATPases [27,39,42]. X-ray crystal structures of folate, hydroxymethyl pyrimidine and pantothenate transporters show that EcfT consists of eight α -helices [38,39,46]. The N-terminus of the protein resides in the cytoplasm whereas its C-terminus is present in the periplasmic compartment. In the membrane, EcfT folds into a L-shape structure with one arm formed by five trans-membrane helices (H1-4 and H8) and the other arm formed by the periplasmic segment consisting of α -helices 5-7 [38,39,46]. This conserved L-shape conformation may be essential for transport activity, by bridging EcfS with the cytosolic ATPases. In addition, the L shape in EcfT is believed

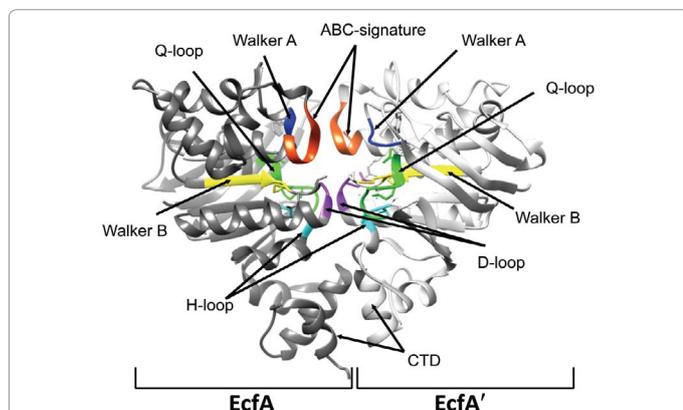


Figure 2: ATPase domains of *Thermotoga maritima* riboflavin transporter.

The EcfA: EcfA' heterodimer is shown. Conserved motifs required for assembly and ATPase activity are highlighted. CTD represents C-terminal domain. Adapted from [27] using PDB 4HLU.

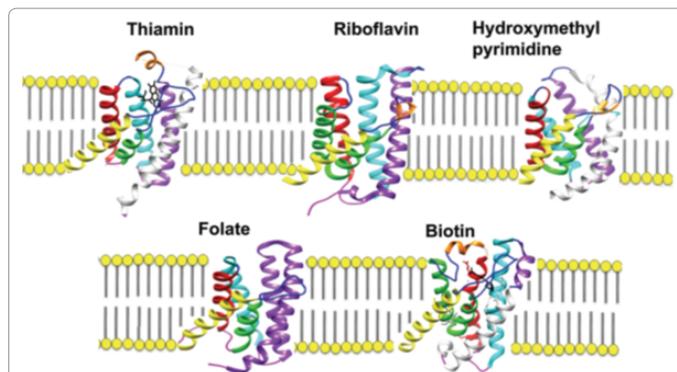


Figure 3: A comparison of crystal structures EcfS- components.

Shown are the structures of the ECF transporters for thiamine (PDB 3RLB), riboflavin (PDB 3P5N), hydroxymethyl pyrimidine (PDB 4HZU), folate (PDB 4HUQ) and biotin (PDB 4VDE). Individual helical structures are coloured with yellow (H-1), green (H-2), red (H-3), cyan (H-4), purple (H-5) and light grey (H-6). Internal and external loops were highlighted in magenta and blue, respectively whereas a short external helix that may function as a putative lid is coloured orange.

to facilitate EcfS to rotate around it, thereby allowing opening of the binding pocket to catalyse substrate capture and transport [38].

Substrate component

The ABC importers require a SBP to recognise and capture appropriate substrates [19]. The high affinity SBP is also responsible for delivering substrate to the open gate of the membrane channel formed by the TMD subunits [43]. In contrast to ABC importers, ECF transporters employ a membrane embedded EcfS subunit to recognise, bind and then transport appropriate substrates into the intracellular environment of the cell [26,42,47]. In other words, the EcfS component possesses both SBP and TMD functions. The EcfS subunits are 20-25 kDa hydrophobic proteins that show low (<25%) sequence conservation between homologues and orthologues [37,48]. Rodionov and co-workers have identified up to 21 coding regions for EcfS components in bacterial genomes [28]. Examples of those that have been functionally characterised include BioY (biotin), CbiMN (cobalt), NikMN (nickel), RibU (riboflavin), FolT (folate), ThiT (thiamin) and PanT (pantothenate) (Figure 3) [26,28,29,49]. Crystal structures of six EcfS proteins have now been determined (Figure 3). The structures commonly consist of 5-6 trans-membrane helices with both N- and C-termini residing in the cytoplasmic region [37-39,46-48,50]. Structural conservation is observed in their N-terminal trans-membrane helices consisting of short helix 1-3 (ThiT, FolT and HmP) [38,39,46,47] or the short helix 1 and a long V-shaped helix 2 (BioY and RibU) [37,48]. The C-terminal trans-membrane helices are highly variable consisting of the short individual helix 4 and long helix 5-6 (Thi and HmP), the long individual helix 4-5 (RibU and BioY) or a long U turn helix 5 (FolT) [30,36,37,39,46]. A comparison of the crystal structures of the unliganded folate transporter from *Lactococcus brevis* and the folate bound transporter from *Enterococcus faecalis* shows a mobile loop, located between helices 1 and 2, closes over the binding site following ligand binding. This structural data has revealed a gating mechanism in the FolT to control substrate binding and release that may also exist in other transporters that have mobile extra-cellular loops, such as BioY, RibU ThiT [30,36].

Intersubunit Interactions

In the classical ABC transporters, TMDs are arranged side by side to create a transportation pore along the dimer interface [51]. Each TMD

subunit has an intracellular coupling helix that can directly interact with a docking groove on the surface of an ATPase subunit [19]. The Q-loop in the ATPase contributes residues to the binding interface [19]. As a consequence, the complex mediates coupling of ATP derived energy production via the NBD subunits with substrate transport in the TMD proteins [23]. NBD subunits in the dimer make a head-to-tail assembly so that the Walker A motif of one NBD subunit is located next to the LSGGQ motif on the partnering subunit. In the nucleotide-free state the Walker A and LSGGQ motifs are separated, but come together in a closed conformation in the nucleotide bound state [44]. Many ATPases of bacterial ABC exporters function as homodimers, but most of the eukaryotic equivalents are heterodimers consisting of either one or two active ATPases [31,32]. Whilst one of the NBD subunits is devoid of a consensus sequence crucial for catalysing ATP hydrolysis, it is still required for assembly into a functional ATPase dimer [52]. The biological significance of this is not understood. In the ECF transporters, direct interactions between the ATPase subunits at the dimer interface occur through residues in C-terminal helix-3 of EcfA and residues in C-terminal helix-2 of EcfA', (Figure 2) [27,39]. X-ray crystal structures of folate and hydroxymethyl pyrimidine transporters reveal that the EcfS component sits on the L-shaped cleft of the EcfT (Figure 4) [27,39]. This is different from the side-by-side conformation found in the classical ABC transporters [31,32]. No significant contacts are observed between the EcfS and ATPase components. Therefore, the primary interaction between the two subunits is stabilized through EcfT [19,33]. The EcfT communicates to each ATPase subunit via coupling helices formed by sequences containing two conserved short Ala-Arg-Gly motifs [49] located at the C-terminal end of cytoplasmic helices 6 and 7 (Figure 4) [27,39]. The interaction between the EcfS and EcfT components involves helices 2-3 of EcfS and five helices of EcfT (Figure 4) [27,39]. Here, conserved hydrophobic residues in helices 2 and 3 of the N-terminal domain of the EcfS subunit, containing the highly conserved A $\phi\phi$ A signature (where ϕ is mostly a hydrophobic residue) [30], form hydrophobic and hydrogen bonding interactions with conserved hydrophobic residues in trans-membrane helices 1-4 and cytoplasmic helix 6 of EcfT [27,39]. Hydrophobic residues in helix 6 and loops L3 and L5 of EcfS also participate in the interactions with EcfT [27], enforcing the predicted role of EcfT as a stabiliser of the complex [26,27,39,42,53]. This structural data also provides a molecular

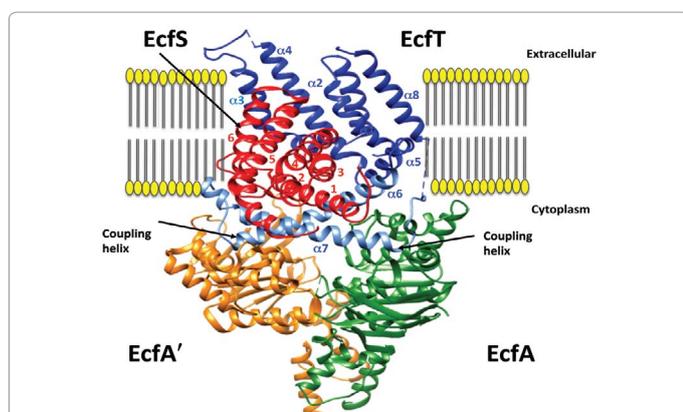


Figure 4: The ECF transporter complex for hydroxymethyl pyrimidine from *Lactococcus brevis*.

EcfS (red) binds to the L-shaped helical region of EcfT (dark blue). Coupling helices α_6 and α_7 of EcfT (light blue) are required for binding the soluble ATPases. The interaction between the EcfA (green) and EcfA' (orange) is facilitated by the C-terminal domains of each protein. Figure adapted from [38] using PDB 4HZU.

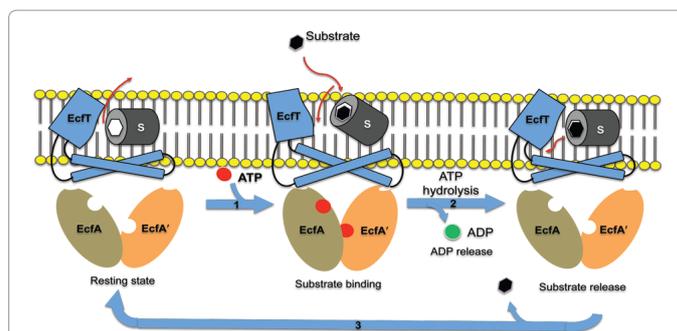


Figure 5: Proposed mechanism of solute uptake.

In this model, EcfS undergoes a 90° rearrangement that permits both binding of substrate in the extracellular environment then delivery of the cargo into the cytoplasm. The energy required for the translocation is provided by ATP hydrolysis. Adapted from [38].

explanation as to how EcfT of Class II transporters can interact with multiple EcfS components.

Mechanism of solute transport

The translocation pore formed by the TMD subunits of ABC transporters is not a continuously open channel [19,23]. This is to avoid potentially lethal substrate accumulation or loss due to unregulated trafficking [51]. Structural data indicate that ABC importers and exporters undergo conformational changes from an ATP-bound open (outward facing) structure to an ADP-bound closed (inward facing) state [31,32,52]. The SBPs use a ligand-induced “clamping” mechanism to uniquely capture appropriate solutes and bring them to the “entry gate” of the translocation pore [43]. Substrate binding at the interface of the globular domains induces rotation at the hinge [19]. A short-lived association between the SBP and translocation pore creates a transporter-bound SBP shuttle that facilitates substrate delivery from the high affinity SBP to the translocation pore [25]. The accepted substrate can then diffuse deeper into the channel created in the dimer interface between the TMDs. Free energy produced during substrate release from the SBP may also enhance substrate movement into the pore. Structural rearrangements in the TMDs during this process are transmitted to the NBDs that then undergo a conformational change ready to bind ATP. Tight dimerization of NBDs upon ATP binding brings the coupling helices closer leading to a conformational change whereby the TMDs move from an inward to outward facing state. This facilitates the movement of the substrate further along the translocation pore [19,31]. Finally, ATP hydrolysis and subsequent dissociation of ADP and inorganic phosphate drive TMDs to adopt the inward facing conformation with the release of substrate into the intracellular environment. Here, two ATP equivalents are utilised per reaction cycle [52]. The mechanism of solute translocation with the ECF transporters has not been as well defined as for the ABC transporters. In fact, it is not clear from the available structural and biochemical data how the translocation pore is created for solute movement. There are two prevailing thoughts as to how the assembly of the ECF transporters relates to their function. Here it is reasonable to consider that there is no single, universally conserved mechanism that is employed by all members of the transporter family. In the first model, it has been proposed that the transport pore resides in the protein interface between the EcfS and EcfT subunits [36]. Hence, solute movement involves both trans-membrane proteins in a mechanism similar to the canonical ABC importers. The second model considers that the EcfS protein alone provides the transport path, and large conformational

changes in the membrane facilitate movement of solute from the extracellular medium into the cytoplasm (Figure 5). The three crystal structures of transporters from *L. brevis* support this model, with the EcfS components all aligning parallel to the membrane and the substrate binding pockets positioned next to the cytoplasmic region of the EcfT subunit [38,39,46]. Biochemical studies using nanodiscs embedded with the biotin transporter from *R. capsulatus* have provided further evidence for this “toppling mechanism” where the S subunit is proposed to undergo a 180° conformational change upon ligand binding [38]. By substituting specific amino acids in the dimer interface (A12, A13 and A17 in the A12AφφφA motif of EcfS and V144 and V147 in coupling helix 2 loop of EcfT) with cysteine and using a thiol-reactive crosslinker spanning only 25 Å in length, the authors observed that the addition of ATP did not disrupt the protein: protein interaction during catalysis [54]. This mechanism is plausible for class I transporters, but is not sufficient to explain the mechanism for the class II ECFs that utilize one shared T component. Recent size exclusion chromatography studies on the riboflavin transporter complex from *Listeria monocytogenes* have addressed this issue [40]. In the presence of ATP this ECF complex dissociates, suggesting that the S subunit is liberated from the EcfT-EcfAA” complex in a form competent to rebind ligand. Upon binding of the riboflavin the transporter complex reforms, and ATP hydrolysis is proposed to drive the 180° conformational change required to move ligand across the membrane and deliver it to the cytoplasm. More data from structural and biochemical studies will further define the various mechanisms underlying solute translocation.

Other biotin transporters

After discussing the ECF biotin transport system utilising BioY in prokaryotes, in this section we compare the biotin transport proteins from other organisms, specifically *E. coli*, mammals, yeast and plants. These organisms all employ uptake systems that are distinct from the ECF system previously described, and are summarised in Table 1. The different examples of high affinity transporters for the same micronutrient reflect the essential and ubiquitous biological function of biotin as an enzyme cofactor found throughout biology. These differences also represent opportunities to specifically design therapeutic compounds that exploit the biotin transporters to permeate biological membranes.

E. coli biotin transporter, YigM

E. coli is a biotin-prototrophic, γ-proteobacterium that is capable of biotin import and export [55,56]. It is unclear why this bacterium has evolved a biotin uptake system that is distinct from the ECF transport system that is widely employed by other bacteria and archaea. Early biochemical studies indicated that biotin uptake occurs via a carrier-facilitated, high affinity and energy dependent mechanism [57]. Although a number of studies have been performed over the past 30 years to characterise this uptake system, the protein responsible has only recently been identified [58]. The YigM protein is a 299 amino acid protein (MW ~34 kDa) consisting of 10 trans membrane domains. This protein is not structurally related to BioY, but instead belongs to the carboxylate/amino acid/amine family of secondary transporters [58]. There is no structural data for the YigM protein, and it shows no sequence homology with either the yeast or mammalian biotin transporters. YigM actively mediates substrate acquisition using facilitated diffusion with high affinity for biotin (K_M of 74 ± 14 nM) [58]. The energy source that drives uptake is unclear, but iodoacetate (inhibitor of glycolysis) and azide (inhibitor of electron transport) both inhibit biotin uptake [57], implying a role for ATP hydrolysis. The

Name	Species	TMD ¹	Structure and higher order complex	Energy Source and Specificity
Class I Energy Coupling factor BioY	<i>Rhodobacter capsulatus</i> , <i>Chlamydia trachomatis</i>	12	Complex with ECF T-AA” Discrete permease and ATPases	ATP hydrolysis
Class II Energy Coupling factor BioY	<i>Corynebacterium glutamicum</i>	12	Complex with ECF T-AA” Shared permease and ATPases	ATP hydrolysis
YigM	<i>Escherichia coli</i>	10	Not known	Possibly ATP hydrolysis
VHT1	<i>Saccharomyces cerevisiae</i> <i>Schizosaccharomyces pombe</i> .	12	Not known	Proton dependent symporter.
SMVT	<i>Homo sapiens</i>	12	Not known	Sodium dependent, diffusion using membrane gradient. Also transports pantothenic acid and lipoic acid.
MCT1	<i>Homo sapiens</i>	12	Not known	Proton dependent, counter transport. Also transports lactate, acetate, β-hydroxybutyrate and hexanoate.

¹Number of trans-membrane domains.

Table 1: Summary of biotin transport proteins.

prominent role of YigM for biotin transport has been demonstrated using a mutant strain of *E. coli* harbouring the mutant protein YigM S28R, P243S defective in biotin transport [58]. However, YigM is not the only possible route used by this bacterium to facilitate biotin acquisition as *E. coli* defective in biotin biosynthesis and YigM are still able to grow in medium containing high concentrations of biotin [58]. This nonspecific transport might be possible through alternative trans-membrane proteins, such as aquaporins.

Mammalian Sodium -dependent multivitamin transporter (SMVT1)

As mammals are unable to synthesize biotin *de novo*, organisms in this class must satisfy their demand for the micronutrient by uptake from exogenous sources. Hence, a high affinity transporter is essential to provide cells with sufficient co-factor to sustain the activities of the five biotin-dependent enzymes. SMVT1 is the primary transport protein that mediates biotin import into mammalian cells. This transporter utilises a trans-membrane sodium ion gradient and membrane potential to drive active translocation of biotin (Vitamin B7), pantothenic acid (Vitamin B5) and lipoic acid into the cell [59-61]. SMVT1 has been investigated using intact cells from various mammalian tissues SMVT [59,62-65], membrane vesicles [66,67], and nonmammalian oocytes recombinantly expressing mammalian SMVT [64]. The SMVT1 is a 68,600 Da protein of 636 amino acids [64]. No structural data has been reported for SMVT1, but modelling studies propose it contains twelve TMD subunits. It is not known if the protein forms higher order complexes either with itself or other regulatory proteins. The protein contains three extracellular N-glycosylation sites (N138, N489 and N498) and two intracellular consensus sequences for protein kinase C-dependent phosphorylation at S283 and T286 [64]. Mutation of the glycosylation sites at N138 and N489 reduced the V_{max} of the importer

activity, but did not affect the affinity for biotin, implying a key role of the sugar moieties in the activity of the transporter [68]. Likewise, post-translational phosphorylation of SMVT is utilized to regulate SMVT activity [69]. This was demonstrated in colorectal adenocarcinoma cells where the addition of protein kinase C activators reduced uptake of biotin whereas the presence of kinase inhibitors stimulated its uptake [60]. Primary structure analysis reveals a high degree of sequence conservation between mammalian SMVTs, with amino acid similarity ranging from 89% to 92% [64]. SMVT is widely expressed in various tissues such as intestine, liver, brain, heart, lung, kidney, cornea and placenta [70]. Therefore SMVT has the potential to be exploited as vehicle in drug delivery, as will be discussed later.

Mammalian monocarboxylate transporter (MCT)

MCT1 has also been proposed as an alternative mammalian biotin transporter in cells of the lymphoid lineage [71], despite these cells also expressing a functional SMVT [69]. In mammalian colonic luminal membrane vesicles, MCT1 acts as the main protein that facilitates translocation of bicarbonate ions from the colonic lumen into colonocytes [72]. Similar to the colonic system, MCT1 in human peripheral blood mononuclear cells also facilitates the acquisition of biotin into the cells using a proton-dependent counter-transport mechanism. MCT1 catalyses translocation of biotin across the cellular membrane via the replacement of monocarboxylic acids [71]. As a consequence, biotin uptake is inhibited by monocarboxylic compounds such as lactate, β -hydroxybutyrate, acetate and hexanoate [71]. MCT1 is the only transporter reported to facilitate biotin uptake in human lymphoid cells [71].

Yeast vitamin H transporter-1 (VHT)

The VHT1 is a membrane transporter protein employed by biotin-auxotrophic yeast *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* to import biotin via a proton (H⁺)-biotin symport mechanism [73-75]. Proton dependency of this permease has been confirmed using trans-membrane proton gradient uncouplers [74] and activators [76]. VHT1 consists of 593 amino acids (586 in *S. pombe*) that assemble into 12 trans-membrane helices [74,75]. This transporter contains a long (120 amino acid residues) N-terminal hydrophilic domain, with both N- and C-termini in the cytoplasm [74,75]. In *S. pombe* the trans-membrane helices are segregated into 2 sets of 6 helices by a cytoplasmic hydrophilic loop consisting of 71 residues between helix 6 and 7 [75]. Two extracellular asparagine residues, present in the conserved sequence for N-glycosylation in the *S. cerevisiae* VHT1, N146 (between helix 1 and 2) and N406 (between helix 7 and 8) are proposed to be glycosylated during secretion [74]. No mutational studies have been performed on VHT1 to establish if the glycosylation is essential for activity like it is for SMVT. Biochemical studies show that VHT1 is capable of both biotin import and export. Import is dependent upon temperature, acidity (pH), and intracellular energy levels [76]. Following biotin starvation, biotin uptake reaches a plateau after 20 minutes of biotin re-addition. After reaching a maximum intracellular concentration, biotin efflux occurs in a temperature and pH independent manner [77]. The optimum temperature for uptake was determined to be 30°C, and optimum pH ranged from 3.8 to 4.0 for total or free biotin and 2.8 to 7.0 for protein-bound biotin [76]. Uptake was also controlled by the biotin concentration in the growth medium [77,78]. VHT1 shows high selectivity for biotin and close structural analogues. Using radiolabeled biotin, the K_m was measured to be 3.3 μ M. Uptake of the labeled-biotin was inhibited by non-labeled biotin and analogues such as homobiotin, norbiotin, desthiobiotin,

oxybiotin, biotin sulfon, and biotinyl-p-nitrophenyl ester (BNP), but not by the precursor 7,8-diaminopelargonic acid (DAPA) nor biocytin (N-biotinyl-L-lysine) [75,78]. Although VHT1 belongs to the allantoin permease family it cannot translocate allantoin or other structurally related analogues such as ureidosuccinate and pantothenate [74,75]. VHT1 shows no sequence homology to the mammalian SMVT1 [74].

Plant Vitamin H Transporter-1

A proton-dependent symporter for biotin has been reported from *Arabidopsis thaliana* [79]. This transporter, encoded by the AtSUC5 gene, is the same transporter responsible for sucrose import. It shows low sequence conservation with both yeast VHT1 and mammalian SMVT1. Unlike yeast VHT1 [74] and mammalian SMVT1 that are inhibited by low concentrations of biotin, plant VHT1 mediates the uptake of the vitamin in a concentration-dependent manner that is not saturable at high concentrations (up to 2 mM) of biotin [79]. Deletion of the suc5 gene caused severe delays in embryo growth and triacylglycerol production in plants that are also deficient in biotin biosynthesis [80]. There has been very little work published on the plant transporters.

Possible Application of Biotin Transporter for Drug Discovery

The biotin transporter can be employed for drug delivery because of its capability to specifically uptake various biotinylated molecules with therapeutic potential. Several studies highlight the utility of this approach, despite the mechanisms for the transport being poorly understood in most cases. Previous research by Walker and Altman reported that Gram-negative *E. coli*, *Salmonella enterica* serovar *Typhimurium* and *Pseudomonas aeruginosa* can import 10 – 31 amino acid peptides, with an average molecular weight of 3,900 Da, once conjugated with biotin [81]. These peptides were much larger than the biggest (~650 Da) non-biotinylated hexapeptide peptide that could be imported by *E. coli*. The distinct translocation mechanisms employed by bacteria and mammals provides a therapeutic window to design antibiotics that are selectively imported by bacteria. The example here also suggests that therapeutic molecules that are larger than typical drug-like, small molecules (molecular weight <500 Da) is achievable. This is a welcome finding given the significant amount of research activity into developing peptide-based antibiotics. Furthermore, novel approaches that improve the permeability of difficult to treat Gram-negative bacteria may rekindle interest in promising compounds that were discarded from antibiotic discovery projects due to poor cell penetration activity. Bacterial and yeast systems represent potential targets for the delivery of new anti-infectives with applications in medicine and agriculture. Transporter targeted delivery has become a powerful approach to deliver drugs to target tissues. One noteworthy example is acyclovir, a first line drug for the treatment of herpes simplex virus infection of the eye. Various strategies have been employed to improve the cellular permeability of this compound, including synthesizing lipid prodrugs. Vadlapudi *et al.* generated a biotinylated analogue of acyclovir to enhance cellular absorption in the cornea [82]. Cellular uptake assays showed that the biotinylated-lipid prodrug was a significantly improved substrate over the nonbiotinylated drug. The synergistic improvement in bioactivity was proposed to be due to the binding of the prodrug to SMVT through the biotinyl moiety and increased lipid mediated cellular uptake into cells.

SMVT is overexpressed in a number of cancers [83], an observation that can be exploited for the delivery of novel anticancer drugs. Camptothecins, that target topoisomerase, are effective anti-

cancer agents but have significant side effects that limit the dose that can be safely administered and, therefore, anti-tumour efficacy. Multiple analogues are in development to improve the safety and efficacy of this drug class [84]. Chemical modifications in the form of biotinylation and PEGylation are amongst the approaches that have been explored. Improved cytotoxicity of biotinyl-PEG-camptothecin against drug sensitive and drug resistant ovarian carcinoma cell lines has been reported [85]. The improved efficacy of the treatment was due to enhanced SMVT mediated uptake. Similarly, biotinylation and PEGylation of the anti-HIV protease inhibitor saquinavir has improved the pharmacokinetics of this drug through by improved cellular permeability and inhibition of P-glycopeptide mediated efflux [86]. Given the enormous potential of biotin-mediated approaches for drug delivery, more research is required to better understand substrate specificity and mechanism of biotin transporters in various species.

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