Energy coupling in ABC exporters

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Abstract

Multidrug transporters are important and interesting molecular machines that extrude a wide variety of cytotoxic drugs from target cells. This review summarizes novel insights in the energetics and mechanisms of bacterial ATP-binding cassette multidrug transporters as well as recent advances connecting multidrug transport to ion and lipid translocation processes in other membrane proteins.

Keywords

Bioenergetics; ABC transporter; Multidrug transport; Transport mechanism

Introduction

With contributions from biochemistry, biophysics, cell biology, chemistry, genetics, microbiology, oncology, pathology, pharmacology, pharmacy, physiology and structural biology (in alphabetical order) and close to 500,000 published research papers in PubMed since the first publication on penicillin resistance in 1940 [1], drug resistance is one of the largest, most interesting and relevant areas of research of this era. Antibiotics and cytotoxic anticancer drugs transformed human and veterinary medicine, but we have reached a point where there are hardly any treatment alternatives left for some drug-resistant bacterial infections and where 50% of cancers develop some form of drug resistance during treatment [2-4]. Cytotoxic drugs act by exerting toxic actions within the cell. One important mechanism of drug resistance is therefore based on the extrusion of cytotoxic agents by multidrug transporters from the cellular interior to the external environment. Due to this extrusion, the cytoplasmic drug concentration is lowered to subtoxic levels, allowing the cell to overcome drug toxicity. In the sections below, we will summarize basic properties of ATP-binding cassette (ABC) multidrug transporters and their sources of metabolic energy with emphasis on, but not restricted to, bacterial homodimeric proteins. Recent data will be discussed that narrow the apparent gap between ABC multidrug transporters and classes of ion-coupled multidrug transporters. To facilitate this discussion, we will start with a brief introduction into bacterial bioenergetics.

Bacterial bioenergetics

The nutrient and energy demands of microorganisms are frequently not fully met in natural habitats. Although some solutes are readily available to living cells in the aqueous environment, many other nutrients are very scarce and not easily obtained. Moreover, microorganisms are exposed not only to changes in chemical gradients of nutrients but also to fluctuations in temperature, pH, redox potential and osmolality. In contrast to the ever changing outside world, the chemical and physical composition of the interior of microorganisms is much more constant to optimally support the biological processes and metabolic pathways underlying DNA replication and cell proliferation. Cells are able to maintain this difference between internal and external environments due to the presence of a phospholipid bilayer, referred to as the cytoplasmic membrane, which provides a barrier that slows down the rapid diffusion of solutes and ions. The cytoplasmic membrane contains channels and transport proteins that facilitate the uptake of nutrients and ions required for growth and metabolism at physiological rates exceeding the rates of diffusion. Membrane proteins also facilitate the efflux of end products of metabolism and of cytotoxic agents encountered in the environment.

In bacteria, metabolic energy transducing processes in the cytoplasmic membrane lead to the generation of ion motive force and the formation of ATP.

Membrane transport proteins belong to a limited number of protein families. They can generally be classified into primary-active transport systems that utilize phosphate-bond energy in the form of nucleotides to drive substrate transport against a concentration gradient, and secondary-active transporters that transduce one form of electrochemical energy into another form [5]. Energetically, the most-important electrochemical ion gradient across the cytoplasmic membrane in many bacteria is that of protons [6, 7]. Redox-driven proton pumps in respiratory chains in aerobic bacteria and the H^+-ATPases in (facultative) anaerobic bacteria generate these electrochemical proton gradients. The outwardly-directed translocation of protons and their positive charge results in the generation of two components: a chemical proton gradient (ΔpH, inside alkaline versus outside acidic) and a membrane potential ($\Delta\psi$, interior negative). These components impose an inwardly-directed force on the protons referred to as the proton motive force (Δp) that equals: $\Delta\psi$ - ZΔpH mV, in which Z equals 2.3RT/F (where R is the gas constant, T the absolute temperature and F the Faraday constant). Secondary-active membrane transporters transduce the metabolic energy contained in the Δp into (electro)chemical energy of substrates by mediating the influx of protons across the cytoplasmic membrane in co-transport (symport) or counter transport (antiport) with substrates. The metabolic energy released during this movement of protons is captured in the transporters and used to drive the transport of substrates against their concentration gradients. Although proton cycling plays a central role in energy transduction in many living cells, sodium cycling is also frequently observed, particularly across cytoplasmic membranes in thermophilic bacteria, eukaryotic microorganisms and mammalian cells. The sodium motive force (Δp_{Na}, interior negative and low) is defined in an analogous way to the Δp, and often coexists with the Δp in mesophilic bacteria.

Families of multidrug transporters

An interesting class of membrane transporters is that of the multidrug transporters. Some of the best-studied systems are ATP-binding cassette (ABC) transporters that utilize ATP binding and hydrolysis as a source of metabolic energy in the drug translocation process. Other multidrug transporters are member of the Major Facilitator Superfamily (MFS), Multiple Antibiotic and Toxin Extrusion (MATE) family, Proteobacterial Antimicrobial Compound Efflux (PACE) family, or Resistance-Nodulation-cell Division (RND), and are ion-coupled

transporters that are dependent on electrochemical ion gradients [8]. While the majority of membrane transporters exhibit a selectivity for one substrate or class of substrates, multidrug transporters have the ability to transport a very wide range of structurally dissimilar substrates. These substrates often have amphiphilic properties allowing their adsorption onto membrane-water interfaces, from where they diffuse across cytoplasmic membranes into the interior of cells. This is particularly relevant for many therapeutic drugs including antibiotics and cytotoxic anticancer drugs that accumulate at the inner membrane leaflet of the cytoplasmic membrane by $\Delta\psi$ (interior negative)-driven passive uptake of the permanent cation or protonated weak base, or by ΔpH (interior alkaline)-dependent trapping of the weak acid following its deprotonation in the cellular interior. Many multidrug transporters belonging to ABC and MFS families, and possibly also the MATE and PACE families, have the ability to transport amphiphilic drugs from the inner membrane leaflet into the compartment that is external to the cytoplasmic membrane. In Gram-negative bacteria, this compartment is a gel-like matrix (referred to as periplasm) that is separated from the extracellular environment by the outer membrane. Due to the presence of lipopolysaccharides in its outer leaflet, the outer membrane provides another permeability barrier that slows down the diffusion of amphiphilic antibiotics into the cell. The transporters in the ABC, MFS, MATE and PACE families therefore act in conjunction with multidrug tripartite transporters of the RND family, which can transport drugs from the outer leaflet of the cytoplasmic membrane and periplasm across the outer membrane to the extracellular environment. In this way, drug transport across the cytoplasmic membrane and outer membrane are part of the same drug elimination pathways [9]. The phenomenon of drug elimination pathways is also encountered in mammals. Here, the ABC multidrug transporters ABCB1 and ABCG2 (also referred to as multidrug resistance P-glycoprotein and breast cancer resistance protein, respectively) for amphiphilic compounds and the ABC multidrug transporters ABCC1 and ABCC2 (also referred to multidrug resistance-associated protein 1 and 2) for sulfate, glucuronide and glutathione conjugates produced in phase I and II drug metabolism, work together to direct incoming amphiphilic drugs in the gut and other locations towards their excretion by the liver and kidney [10]. In summary, cells generate drug permeability barriers that limit drug diffusion into the cell, and they express multidrug transporters that catalyse drug efflux across these barriers at rates that outcompete the rates of diffusive uptake. This ingenious system allows the cell to effectively eliminate drugs from the interior. In the repertoire of available drug resistance mechanisms in microorganisms, drug efflux is therefore an important intrinsic first-line of defence of cells

against cytotoxic drugs due to the expression of multidrug transporters at basal levels in unchallenged cells.

Mechanisms of ABC multidrug transporters

Genetic and structural analyses revealed a common architecture of ABC multidrug transporters comprising two highly conserved cytosolic nucleotide-binding domains (NBDs) and two distinct and evolutionary unrelated α-helical membrane domains (MDs) that constitute the substrate-binding chamber and form the transmembrane drug translocation pore [8, 11]. In homodimeric ABC transporters, both NBDs are able to hydrolyse the nucleotide, whereas in heterodimeric ABC transporters usually only one of the ATP-binding sites has the consensus sequences necessary for hydrolytic activity. For most ABC transporters, the bound nucleotide is ATP but there are multidrug transporters that prefer GTP over ATP [12]. Resolved crystal structures [13-15] and cryo-EM structures [16, 17] for homodimeric MsbA, Sav1866 and others (reviewed in [18-21]) provided a significant advancement in our understanding of how multidrug ABC transporters mediate their transport activity. These structures have shown the relevance of basic "alternating access" models in which the transporter cycles between inward-facing and outward-facing states in response to nucleotide binding and hydrolysis. It is often thought that all protein structures generated by crystallography or cryo-EM fit into a generic transport cycle of ABC exporters, and that those that do not fit should be excluded as detergent artefacts. However, even within the class of homodimeric ABC exporters it is very likely that some conformations are only relevant for a subset of transporters, and hence, that transport cycles can differ in the details of transitions [22].

Many studies report on substrate-stimulated ATPase activities for ABC transporters [23]. Recent advances have shed some light on the question of how ATP binding and substrate binding are linked. Studies on MsbA based on cysteine cross-linking, fluorescence energy transfer, and cysteine accessibility indicate that the binding of drugs or the physiological substrate Lipid A stimulates the maximum rate of ATP hydrolysis by facilitating the dimerization of nucleotide-binding domains, thus leading to protein conformations that promote ATP binding and occlude the transport substrate [24, 25]. Investigations on the mammalian MsbA homologue ABCB1 suggest that ATP binding is also followed by ATP occlusion in the NBDs to enable hydrolysis of nucleotide [18, 26, 27] at alternate nucleotide binding sites by structurally asymmetric NBDs [28]. This is further supported by early biochemical observations on the existence of an ATP plus ADP-bound NBD dimer and the

alternating hydrolysis of ATP at the two nucleotide-binding sites [29]. Most recently, the substrate-induced acceleration of ATP hydrolysis in ABCB1 was found to correlate with the stabilization of a high-energy, post-ATP hydrolysis state characterized by structurally asymmetric nucleotide-binding sites [30]. It is still unclear whether the MsbA dimer hydrolyses the two ATP molecules in a random, sequential or alternating fashion. However, for each of these possible mechanisms, asymmetry would develop following the hydrolysis of the first ATP. The *in vitro* selection of designed ankyrin repeat proteins (DARPin) through their specific binding to detergent-solubilized ABC proteins [31] detected asymmetry in the MDs of the MsbA dimer [32]. This asymmetry has not yet been captured in protein crystals or recent cryo-EM structures, and it is therefore not known whether this asymmetry also extends to the NBD dimer.

Structural data indicate that the conformational power delivered by ATP binding and hydrolysis at the NBDs is transmitted to facilitate substrate binding and translocation by the MDs [21]. This is achieved by intracellular loops in the MDs, referred to as coupling helices, that interact with the NBDs. Whereas coupling helix 1 (CH1 connecting TM1 and 3) contacts the NBDs in both half-transporters, CH2 (connecting TM4 and 5) only interacts with the NBD of the opposite half-transporter. These arrangements are different from those in ABC importers and the ABC engine MacB of the tripartite macrolide efflux pump MacAB-TolC, where the MD only contacts the NBD within the same half-transporter [33]. To enable these interactions the NBDs expose short sequence motifs, the Q-loop (in *E. coli* MsbA 424-QNVHLFNDT) and X-loop (in *E. coli* MsbA 473-VIGENGVLL preceding the ABC signature motif), of which the latter is conserved in ABC exporters only. Substitutions of the conserved glutamine residue in the two Q-loops in ABCB1 gave rise to a mutant transporter that is trapped in the inward-open state; the mutant binds drug but cannot couple to the ATPase cycle [34]. In the human phosphatidylcholine ABC transporter ABCB4, a substitution of the conserved glutamine residue in the second X-loop is associated with progressive familial intrahepatic cholestasis type 3 (PFIC-3) in patients with liver disease; the mutation abrogates substrate-stimulated ATPase activity [35]. Recent studies in the MsbA homologue BmrA from *Bacillus subtilis* have shown that, upon ATP-vanadate trapping, substitutions of the conserved glutamate residue in the X-loop impair the transition to the outward facing state [36]. Thus, mutations of conserved residues at the transmission interface between NBDs and MDs often lead to partial or complete inactivation of transport activity. In the absence of a sufficient number of identified intermediates, the currently available protein structures do not yet provide a complete picture

of the intricate inter- and intra-molecular movements that couple metabolic energy to the efflux of drugs.

MDs of ABC transporters show little sequence conservation, which is thought to support the notion that different members of the ABC superfamily recognize and translocate different substrates [37]. However, the lack of sequence similarity is also observed among ABC multidrug transporters with overlapping multidrug recognition capabilities, or when ABC multidrug transporters are compared to ion-coupled multidrug transporters with a similar drug selectivity. This implies that the binding of drugs by reversible weak interactions can be achieved through contributions of different subsets of side chains in drug binding pockets. Structural analysis of drug-bound transcriptional regulators [38, 39], RND systems [40, 41], ABCB1 [42, 43] and ABCC1 [44] confirm this conclusion. Equilibrium vinblastine binding data for the MsbA homologue LmrA from *Lactococcus lactis* demonstrated for the first time the binding of two vinblastine molecules with high and low affinity [45]. This protein-drug ratio is also observed in cryo-EM structures of MsbA in which two quinolone inhibitor G907 molecules are bound in architecturally-conserved side pockets that accompany the central binding chamber [17]. Moreover, crystal structures of mouse ABCB1a exhibit the binding of two molecules of the cyclic peptide inhibitor QZ59-SSS in the central binding chamber [42], and analogous cryo-EM studies with a hybrid construct of human and mouse ABCB1 demonstrate the binding of two molecules of the inhibitor zosuquidar in this location [43]. Careful biochemical equilibrium binding experiments, however, indicated single binding sites for zosuquidar in human ABCB1 [46] and vinblastine in Chinese hamster ABCB1 [47], and suggested complex allosteric interactions between multiple drug interaction sites through which the interaction of drug at one site can switch other sites between high- or low-affinity conformations [47]. As ligand concentrations were at least 100-fold higher in the structural studies compared to the equilibrium binding studies, it is conceivable that the presence of a second zosuquidar molecule in ABCB1 remained undetected in the binding experiments if this binding event would occur with a low affinity that is too close to non-specific binding. However, dissimilarities in experimental outcomes could also be due to other major differences in the experimental conditions and sources of ABCB1 protein. The structural data raise the question of how the binding of non-transported inhibitors and transported substrates can have different outcomes on the activity of ABC transporters when these ligands bind in similar pockets and share similar weak drug-protein interactions.

Another feature of most MDs in ABC transporters is the presence of the amphipathic N-terminal elbow helix that runs parallel to the inside surface of the membrane. By analogy to

the peripheral stalk in rotary ATPases that counteract the rotation tendency of the catalytic core in response to the movement of the rotor [48], the elbow helix might firmly associate ABC exporters to the plane of the membrane and counteract rotational movement perpendicular to the membrane. Furthermore, the elbow helix might ensure proper alignment of transmembrane helix (TM) 1 with other TMs in the MD in the outward-facing state in which TM1 and TM3 define the extent of the external opening of the central drug binding pocket towards the external environment [49]. TM1 also contains residues important for regulating the gating of the central drug binding pocket towards the exterior in the inward-facing conformation [50]. Consistent with the importance of the elbow helix, mutant proteins of LmrA with lysine to glutamate substitutions in this helix are properly expressed but have strongly reduced transport activities [51].

Ion transport reactions

Most investigations on transport mechanisms of multidrug transporters have been done from the perspective that ABC transporters have conserved energetic requirements that are distinct from those of secondary-active transporters. The experimental approaches for each of the classes is therefore usually dictated by what is custom in the field. However, interesting observations are made when tools and techniques used in studies of secondary-active transporters are applied to ABC transporters. Reversible substrate transport is a distinct property of secondary-active transporters, and occurs when electrochemical ion gradients driving substrate transport are dissipated. The transport direction is then dictated by the prevailing substrate concentration gradient. One of the consequences of the dissipation of the ion motive force in fermentative bacteria is that ion-translocating ATPases that generate the ion motive force will consume the available Mg-ATP in an attempt to compensate for the loss of ion motive force. In lactococcal cells with a 1000-fold reduction in Mg-ATP concentrations to lower μM levels, ABC exporters such as LmrA, MsbA, and Sav1866 [52-54] exhibit reversibility in drug transport suggesting that the substrate translocation pathway in their MDs is intrinsically reversible. The unidirectionality of these ABC exporters under physiological conditions is therefore at least in part imposed by the exothermic nature of the ATP hydrolysis reaction causing it to be strongly shifted to the ADP + Pi side. More recently, reversibility in transport was reported for the ABC peptide transporter ABCB2/3 (also referred to as TAP1/2) following the substitution of the conserved aspartate in the NBDs in the D-loop that coordinates NBD dimerization [55]. The reversibility of substrate translocation pathways offers the

promise that drug efflux pumps can be utilised for inhibitor-induced drug accumulation in target cells.

Transport studies with ethidium, erythromycin and chloramphenicol in intact cells and proteoliposomes prepared from *Escherichia coli* phospholipids and egg yolk PC established that ATP-dependent drug transport by MsbA is assisted by apparent drug–proton antiport in a reaction that is driven by the inwardly directed ΔpH (interior alkaline) [56]. Interestingly, ethidium transport by this ABC transporter was also stimulated by a reversed Δψ (interior positive in cells) rather than the physiological Δψ (interior negative in cells). These findings indicate electrogenic *n*-ethidium$^+$/H$^+$ antiport in which n > 1 but, given the electrophysiological results obtained for LmrA (see below), could also indicate a more complex transport mechanism involving the translocation of additional ions. Proton coupling is associated with the MD dimer of MsbA as the antiport reaction is also observed for a truncated version of MsbA lacking the NBD (MsbA-MD). Furthermore, complete inhibition was observed by inactivating mutations in MsbA-MD (e.g. the triple mutant D41N-E149Q-D252N) [56]. As the MsbA-ATPase activity is stimulated by the ΔpH [56], ATP dependence and proton coupling must be integrated in a common transport reaction. Our findings for MsbA and MsbA-MD are reminiscent of previous studies on LmrA-MD which demonstrated apparent ethidium-proton symport [57]. Most recently, we have extended these studies to full-length LmrA in cells and proteoliposomes, and used electrophysiological studies on LmrA in planar lipid bilayers and giant unilamellar liposomes to characterise the stoichiometry of the ion transport reaction in detail [58]. The efflux of one ethidium$^+$ and one H$^+$ by LmrA occurs in symport with one Cl$^-$, in counter-transport to two Na$^+$, in an electrogenic reaction that is driven by the inwardly-directed sodium motive force in cells (interior negative and low). Remarkably, the ion exchange reaction at the MDs is integrated in the catalytic cycle of nucleotide binding and hydrolysis by LmrA. The reaction is activated in the ATP-bound state of LmrA and is terminated in response to ATP hydrolysis. This type of regulation shares analogy to the ATP regulation of gating in the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) ABC Cl$^-$ channel (also referred to as ABCC7) [59]. Although mutations in NBDs of LmrA and MsbA that inactivate ATP hydrolysis (e.g. deletion of lysine residue in Walker A in MsbA-ΔK382 and LmrA-ΔK388) inactivate ethidium transport in metabolically-active cells [53, 56, 58], both deletion mutants reconstituted in proteoliposomes are transport-active in the presence of appropriate electrochemical ion gradients. This suggests that, when nucleotide is present, nucleotide hydrolysis is compulsory for transport activity to prevent nucleotide trapping of

LmrA. These findings present ion coupling as a novel factor in the transport reaction for LmrA and MsbA. The ability to transport inorganic ions has also previously been observed for secondary-active multidrug transporters. Elegant studies with the *E. coli* MFS multidrug/proton antiporter MdfA demonstrated the ability of this system to mediate K^+/H^+ antiport and to confer alkali tolerance on the cell via MdfA-mediated proton import at the expense of the outwardly directed K^+ gradient [60]. By analogy, the tetracycline efflux pumps Tet(L) and Tet(K) in *Bacillus subtilis* confer alkali tolerance by mediating $(Na^+)(K^+)/H^+$ antiport as an alternative to tetracycline/H^+ antiport [61]. Thus, even when antibiotics are absent, the selective pressure for the persistence of these multidrug transporters in bacterial cells might relate to their physiological roles in pH_{in} regulation [62]. This conclusion could be relevant for any multidrug transporter with an additional physiological role beyond the transport of multiple drugs.

Importance of NBDs in transport efficiency

The ion motive force-dependence of ethidium transport by MsbA-MD and LmrA-MD raises important questions regarding the rationale behind the presence of NBDs in these ABC exporters. A systematic comparison of proton-coupled erythromycin transport by MsbA-MD versus ATP plus proton-coupled erythromycin transport by wildtype MsbA in a drug resistance assay demonstrated that the full-length protein is more efficient than the MD protein [56]. A similar conclusion was obtained in ethidium transport assays where wildtype MsbA effluxed ethidium against larger inwardly-directed concentration gradients than MsbA-MD. It is interesting to note that a dual mode of energy coupling has also been reported for other transporters; four examples will be discussed. (i) The arsenite and antimonite-translocating ArsB protein from *E. coli* [63, 64] acts as a secondary-active metalloid–proton antiporter, but when associated with the ArsA ATPase subunit also uses ATP to enhance its transport efficiency. This leads to higher levels of drug resistance as compared to the levels obtained with ArsB only. (ii) The SERCA Ca^{2+}-ATPase sequesters Ca^{2+} in the sarcoplasmic reticulum of cardiac and skeletal muscle whereas the PMCA Ca^{2+}-ATPase mediates Ca^{2+} extrusion across the plasma membrane of neurons. These P-type ATPases mediate Ca^{2+}- proton antiport during the ATP-dependent transport reaction to ensure neutralization of the Ca^{2+}-coordinating carboxylates following the dissociation of Ca^{2+} [65-67]. As the proton permeability of mammalian membranes is frequently too high to sustain significant chemical proton gradients, Ca^{2+} transport by these pumps is not known to be regulated by the ΔpH [65]. Thus, primary-

active membrane transporters may translocate inorganic ions as part of their catalytic mechanisms, but the prevailing conditions determine whether (electro)chemical gradients for those ions are of a sufficient magnitude to affect the overall rate of transport. (iii) ATP and proton motive force-dependent transport are observed for protein secretion by the hetero-trimeric membrane protein complex SecYEG in *E. coli* [68, 69]. In this system, phosphate bond energy is captured by the cytosolic motor protein SecA that uses ATP-driven cycles of insertion and retraction from the membrane-bound SecYEG translocon to mediate processive extrusion of preproteins through the cytoplasmic membrane. *In vitro* translocation assays show that the ATPase activity of SecA is required for the transmembrane transport of the NH_2-terminus of the preprotein, which includes the signal sequence that targets proteins for export through the translocon. The ATPase activity of SecA is also required to drive the transport of downstream COOH-terminal segments of the preprotein in the absence of a Δp. However, once preprotein translocation is initiated, the translocation machinery can operate without the input of ATP in a reaction driven by the Δp via the auxiliary complex SecDF [70] and a recently proposed ΔpH-dependent proton ratchet mechanism by SecYEG [71]. Similar to ArsAB and MsbA, the simultaneous input of ATP binding-hydrolysis and the Δp achieves efficient protein secretion. (iv) The type I secretion system HlyBD-TolC in *E. coli* contains the MsbA and LmrA homologue HlyB as the ABC translocator [72]. The extrusion of the secretion competent C-terminal peptide (Actp) of the toxin protein haemolysin A by HlyBD-TolC was reported to be severely inhibited by the proton ionophore carbonylcyanide m-chlorophenylhydrazone (CCCP), which collapses simultaneously the Δψ and ΔpH. Inhibition was also obtained by the addition of K^+ plus valinomycin, a potassium ionophore that disrupts the Δψ [73]. Thus, these data for HlyBD-TolC point to a transport mechanism that is ATP dependent as well as Δp dependent.

Our observations on the ATP and ion-coupling to multidrug transport by MsbA and LmrA might relate to roles of these ABC transporters in the transport of physically large or polymeric substrates via mechanisms that utilize a dual mode of energy coupling to obtain physiologically relevant translocation rates. For MsbA, one such substrate is core Lipid A. Structural studies with cryo-EM have shown the binding of Lipid A in the large chamber at the interface between the two MsbA half-transporters that form the transport-active complex [16]. However, as the pre-flopping conformation (containing Lipid A with head group near the cytoplasmic surface of the inner membrane) and post-flopping conformation (containing Lipid A with head group near the external surface of the inner membrane) show similar interactions

with ATP, it is not yet clear whether ATP is the sole form of metabolic energy that is relevant in Lipid A transport. In this context it is interesting to refer to recent progress on the mechanism of Ca^{2+}-activated lipid scramblase TMEM16 proteins, which were found to contain a membrane-exposed hydrophilic groove that serves as a shared translocation pathway for lipids and ions [74, 75].

Outlook

Multidrug transporters are fascinating molecular machines, many features of which remain to be discovered. In our studies on MsbA and LmrA, we uncovered novel mechanisms of inorganic ion transport in ABC exporters that are not yet predicted in current crystal and cryo-EM structures, and that might be linked to physiological roles of these proteins in the transport of drugs, lipids or biopolymers, or be important in facilitating rate-limiting nucleotide-independent steps in the overall transport reaction. Kinetic schemes for symport or antiport reactions by bacterial secondary-active transporters are usually relatively simple, and are thought to involve the binding and dissociation of transport substrates and H^+ or Na^+ as coupling ion. It is remarkable that kinetic schemes for mammalian secondary-active transporters are often far more complex. Take for example the mammalian excitatory amino acid transporters (EAAT), some of which mediate the re-uptake of synaptically released glutamate into nerve terminals. What started originally as a 'simple' glutamate - Na^+ symporter mechanism based on biochemical assays [76] turned into a more complex co-transport mechanism of three Na^+, one H^+, and one glutamate, in counter-transport to one K^+ when the transporter was studied by electrophysiological methods (reviewed in [77]). Moreover, EAAT transporters contain a Cl^- channel that is thermodynamically uncoupled from glutamate uptake to ensure that the high rates of cation uptake in nerve terminals are not inhibited by the build-up of a reversed $\Delta\psi$ (interior positive) [78]. Traditionally, electrophysiological techniques have been applied to mammalian cells but not to bacteria, which were too small to be patched with glass pipettes. However, recent advances with the overexpression and detergent-based purification and reconstitution of bacterial membrane proteins has brought these transport systems within reach of electrophysiological techniques. Our electrophysiological and biochemical data for LmrA suggest that ion transport in bacterial transporters can have a similar complexity as observed for mammalian transporters. If the ABC Cl^- channel CFTR (ABCC7) evolved from an ABC transporter through loss of function [59], it will certainly be interesting to test which mammalian ABC transporters translocate Cl^- and/or other inorganic ions as part

of their transport activities. In conclusion, significant progress has been made in the structural biology of ABC multidrug transporters, but the proper interpretation of the structural data requires knowledge of all transport activities that are catalysed, as well as insight into the question of how these activities are coupled to metabolic energy. The synergy between structural, biochemical and biophysical studies on purified proteins in nanodiscs, proteoliposomes, giant unilamellar vesicles and planar lipid bilayers, and *in vivo* studies in intact cells therefore holds the promise to answering mechanistic questions regarding these membrane transporters and those in other families.

Acknowledgements

We apologize to all colleagues whose important work could not be cited in this brief review. We thank David Spring and Sam Rowe (Department of Chemistry, University of Cambridge), Mark McAlister, Philip Rawlings and Dominic Williams (AstraZeneca), and Martin Picard, Dhenesh Puvanendran and Bruno Miroux (Institut de Biologie Physico-Chimique, French National Centre for Scientific Research (CNRS)) for discussions. Research associates Himansha Singh and Kelvin Agboh are supported by Biotechnology and Biological Sciences Research Council (BBSRC) grant BB/R00224X/1 and BB/I002383/1 and Human Frontier Science Program (HFSP) grant RGP0034/2013. Dawei Guo is funded by a China Scholarship Council – Cambridge Trust PhD Scholarship. Brendan Swain received an AstraZeneca PhD studentship. Robbin de Kruijf is supported by an Integrative-Toxicology Training Partnership (ITTP) PhD studentship from the Medical Research Council. Charlotte Guffick is funded by a BBSRC Doctoral Training Partnership (DTP) PhD Studentship. Hendrik van Veen is the recipient of the Chaire Edmond de Rothschild 2019 prize from the Edmond de Rothschild Foundation for Scientific Research and Development at the CNRS in Paris.

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