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Review

Structural insights into functional lipid–protein interactions in secondary transporters ☆

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ABSTRACT

Background: Structural evidences with functional corroborations have revealed distinct features of lipid–protein interactions especially in channels and receptors. Many membrane embedded transporters are also known to require specific lipids for their functions and for some of them cellular and biochemical data suggest tight regulation by the lipid bilayer. However, molecular details on lipid–protein interactions in transporters are sparse since lipids are either depleted from the detergent solubilized transporters in three-dimensional crystals or not readily resolved in crystal structures. Nevertheless the steady increase in the progress of transporter structure determination contributed more examples of structures with resolved lipids.
Scope of review: This review gives an overview on transporter structures in complex with lipids reported to date and discusses commonly encountered difficulties in the identification of functionally significant lipid–protein interactions based on those structures and functional in vitro data. Recent structures provided molecular details into regulation mechanism of transporters by specific lipids. The review highlights common findings and conserved patterns for distantly related transporter families to draw a more general picture on the regulatory role of lipid–protein interactions.
Major conclusions: Several common themes of the manner in which lipids directly influence membrane-mediated folding, oligomerization and structure stability can be found. Especially for LeuT-like fold transporters similarities in structurally resolved lipid–protein interactions suggest a common way in which transporter conformations are affected by lipids even in evolutionarily distinct transporters. Lipids appear to play an additional role as joints mechanically reinforcing the inverted repeat topology, which is a major determinant in the alternating access mechanism of secondary transporters.
General significance: This review brings together and adds to the repertoire of knowledge on lipid–protein interactions of functional significance presented in structures of membrane transporters. Knowledge of specific lipid-binding sites and modes of lipid influence on these proteins not only accomplishes the molecular description of transport cycle further, but also sheds light into localization dependent differences of transporter function. This article is part of a Special Issue entitled Structural biochemistry and biophysics of membrane proteins.

1. Introduction

Biological membranes are essential cellular components central to all life processes. They provide a selective and electrochemically sealed permeability barrier for cells and allow compartmentalizing cellular organelles. Proteins embedded in these lipid bilayers mediate transport and communication between the two sides delineated by the membrane. These integral membrane proteins are involved in many crucial life-sustaining processes like respiration, transport and photosynthesis. Not surprisingly, they typically comprise almost 20–30% of the annotated genes of known organisms [1]. It has long been understood that the lipid bilayer surrounding membrane proteins is not just a passive environment but actively contributes to membrane protein properties. For instance, lipids are known to confer structural stability and mediate oligomerization as seen in aquaporins and bacteriorhodopsin [2,3]. They help in the assembly of supercomplexes like cytochrome bc1 [4]. Some membrane proteins require specific lipids as chaperons in topogenesis, e.g., lipids assist in folding and correct insertion as documented in the potassium channel KcsA and lactose transporter LacY [5,6]. In fact, LacY can adopt altered topologies by simply changing the lipid composition of the membrane. Lipids also directly affect and modulate protein function as seen in mechanosensitive channels MscL [7] responding to hypoosmotic

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stress. Bilayer adjustments to alleviate hydrophobic mismatch gate the opening of inward rectifying potassium channels K_{ir} where binding of specific signaling lipid phosphatidylinositol-4,5-bisphosphate (PIP2) controls channel opening (reviewed in detail in [8]). Identification of lipid-binding sites in membrane proteins that are involved in human diseases has led to the development of membrane–lipid therapies with specific lipid–protein interactions being increasingly used as therapeutic targets in molecular medicine [9].

Lipid–protein interactions can be either of chemical nature when individual amino acids in proteins coordinate individual lipids, thereby forming a specific binding site or of physical nature when properties of the bulk lipids, e.g., fluidity, membrane tension, curvature or polarity, affect the protein collectively (Fig. 1a). These chemical and physical properties of lipids are well described, although mostly in artificial systems, it is still unclear how lipid interactions specifically affect protein function at a molecular level. Ultimately it remains difficult to distinguish experimentally between the effect of the lipid bulk and the action of an individual lipid in biological membranes [10].

The complexity of these interactions makes probing using standard structural biology methods non-trivial. Notwithstanding their tremendous importance, molecular details of these interactions are known only for few membrane proteins. Roughly 11% of known membrane protein structures revealed lipid densities (derived from the Membrane Protein Structure Database http://blanco.biomol.uci.edu/mpstruc/, Stephen White). The major holdups in identifying functional lipid interactions in protein structures are the dynamic nature of these interactions, coupled with the modest resolutions usually obtained for membrane proteins. Purification and crystallization procedures also deplete weakly bound lipid moieties. Sometimes even when structural evidences for lipid interactions are present in structures, deducing their functional effects is challenging. On the other hand their small dimensions make membranes and the embedded proteins impossible to image using standard fluorescence microscopy approaches. One bottleneck is therefore to bridge the gap between cellular processes; biochemical/biophysical data on recombinant, often heterologously expressed membrane proteins and structural data.

The understanding of lipid–protein interactions and how they control cellular locations, conformations and the activity of membrane proteins was the motivation to develop new tools for lipid research. Improved imaging techniques such as structured illumination microscopy (SIM), stimulated emission depletion microscopy (STED) and photo-activated-localization microscopy (PALM) emerged to break the diffraction barrier and allow imaging of cellular structures far below the conventional 200 nm limit [11]. Structural information on lipid–protein and protein–protein interactions observed in membrane mimicking environments like two-dimensional crystals was exploited also by spectroscopy, e.g., FT-IR. Techniques that specifically include lipids into the 3D crystallization process [12] have been introduced and successfully applied for receptors, channels and recently also for transporters. Hereby, membrane proteins were either maintained in a lipidic environment during extraction and purification or re-lipidated in bicelles, in lipid cubic phase (LCP) or crystallized in the presence of high concentrations of lipids and detergents (HiLiDe) [13]. The number of structures solved by LCP or derivative techniques like lipid sponge phase (in which the cubic phase is modified by hydrophobic additives) is constantly increasing since the high-resolution structure of bacteriorhodopsin [14]. According to (http://cherezov.scripps.edu/structures.htm) structures of 47 membrane proteins were solved in lipidic phases, 5 of them being transporters (see Section 3). However it is important to note that crystallization in the presence of lipids has not necessarily resulted in the observation of lipids in those structures. Often lipid sites are occupied by detergent molecules (Fig. 1b and c), which although

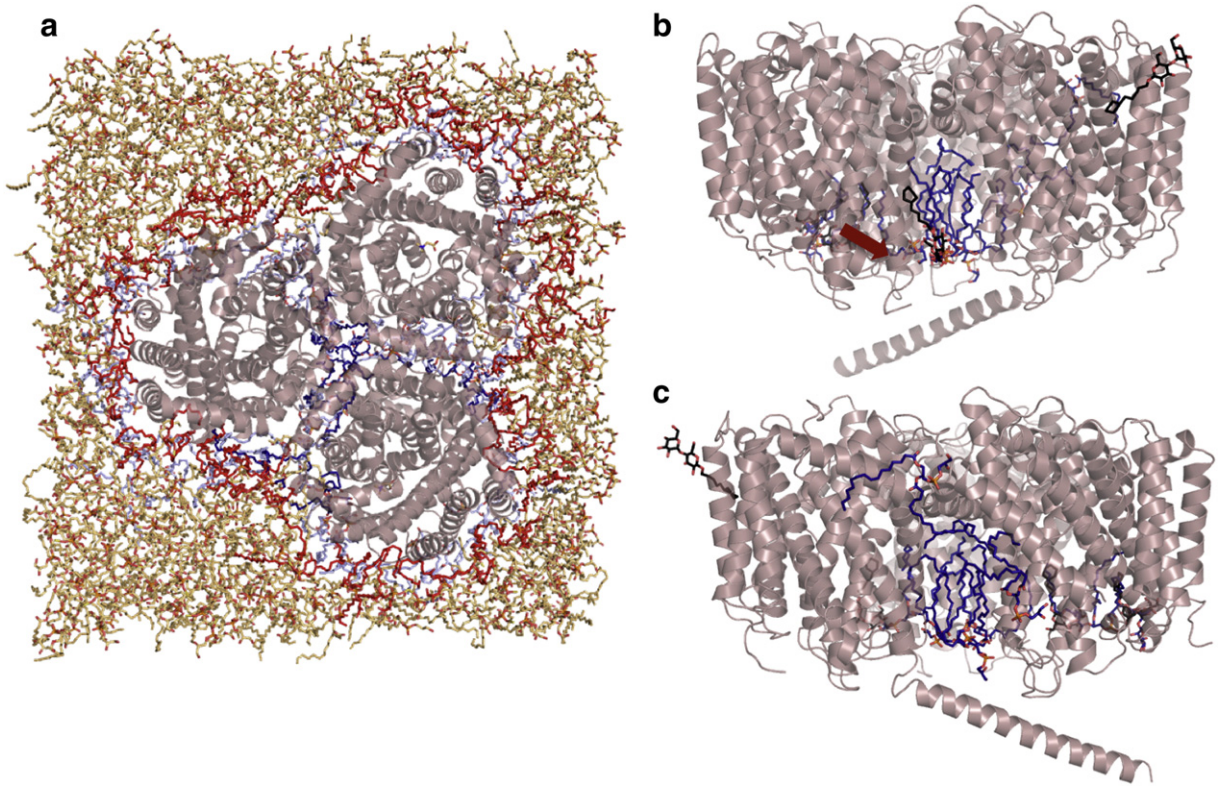

Fig. 1. a) Top view of the X-ray structure of the betaine transporter BetP (PDB: 4C7R) embedded in a hydrated, POPG bilayer. Bulk lipids are depicted in yellow, lipids in direct contact with the transporter trimer are colored in red and specifically bound POPG lipids observed in the crystallographic data are colored in blue. Bilayer water molecules are omitted for clarity. b) and c) Side views of two BetP protomers within the trimer in complex with anionic POPG lipids. The crystallization detergent CYMAL-5 is depicted in black. The red arrow highlights a detergent position, which is next to a lipid observed in another structure of BetP. The detergent positions also align well with the membrane limits like the resolved head groups indicating a possible lipid-binding site position in vivo.

mimicking the hydrophobic interactions of lipids fail to do so for the bulk lipid properties. Therefore, structural information corroborated by functional data for lipid interactions in channels, transporters and receptors have only slowly accrued [15–18].

2. Specific lipid–protein interactions versus bulk lipids in secondary transporters

2.1. Lipid bilayer revisited from the angle of a membrane protein

Our view on the cellular membrane has changed significantly since its description by the fluid mosaic model [19], which depicted the lipid bilayer as a simple two-dimensional fluid in which integral membrane proteins freely diffuse. In fact, lipids and membrane proteins are not homogeneously or randomly scattered within the membrane. Lipid distribution is regulated and laterally organized via active mechanisms that sort both proteins and lipids [20]. Organelle and plasma membranes contain an extremely high amount of proteins, which would not allow their free diffusion in the sense of the fluid mosaic. The dense packing of transmembrane domains from integral membrane proteins imposes an intrinsic order on the "lipid fluid", which is now more adequately considered as "lipid–protein composite" [21].

The presence of ordered membrane protein conglomerates certainly affects the diffusion rate of the lipids themselves, while the flipping of lipid species from one side of the membrane to the other requires the presence of lipid transport proteins [22,23]. As a consequence, processes such as transport across the plasma membrane are vectorially exploiting this lateral and vertical heterogeneity [10]. The concept of ordered lipid phases has culminated in the controversial discussion on the existence of lipid rafts, which hypothesize a liquid-ordered phase formed from saturated phospholipids and sphingolipids in the presence of cholesterol that co-exists with the liquid-disordered phase [24], formed mainly from unsaturated phospholipids [11,25]. It can be assumed that individual integral membrane proteins most likely favor only one of these phases, which would result in their lateral sorting and concentration. However, the lack of direct cellular imaging proof still questions the lipid raft hypothesis although the existence of heterogeneous, highly dynamic, sterol and sphingolipids-enriched domains that compartmentalize cellular processes is commonly accepted [24].

Recently, similarities in membrane order properties, e.g., fluidity, were reported for bacterial, plant, mammalian and fungal membranes that would support to some extent a common concept on how integral membrane proteins exploit the physical properties of the membrane regardless of differences in membrane composition between species [26]. This interesting fact places emphasis on a question we have raised before concerning the impact of specific interactions versus effect of bulk lipids on membrane protein function. The conservation of physical properties under different lipid compositions highlights the influence of bulk lipids, however, many functional and mutagenesis data on transporters can only be interpreted in the light of specific interactions signifying that the question "specific lipids versus bulk lipids" remains still elusive.

2.2. How much membrane a molecular transport mechanism needs?

The functional consequences of lipid–protein interactions differ in different membrane proteins; we will focus in the following discussion on the structural evidences available for lipid–protein interactions in secondary transporters. Secondary transport proteins are ubiquitously found across all kingdoms of life. They are seminal to the functioning of an organism [27,28]. These proteins participate in diverse life sustaining processes like 1) controlling the entry of essential nutrients for metabolic functions, 2) extruding toxins, drugs and metabolic end products from the cell, 3) mediating the uptake and efflux of many ionic species in order to maintain ionic gradients and membrane potentials essential for enzymatic activity and generating bioelectricity, 4) facilitating the uptake and release of various signaling molecules like neurotransmitters and hormones that help cells to communicate with each other and bring to effect specific responses to stimuli, and 5) counteracting the deleterious effects of osmotic stress on the cell by mediating uptake of osmolytes, to list a few [27]. Therefore, the importance of transporters in maintaining cellular homeostasis cannot be overestimated. This is also reflected by the fact that transporters constitute one-third of all known drug targets today [29].

Membrane lipids are known to modulate functions of many secondary transporters, although little is known on the exact nature of lipid interactions on a molecular level [8]. In the past the molecular details of the mechanism of secondary transport were mainly deduced from the structure of detergent-solubilized, crystallized proteins. However, controversial functional data were elucidated that questioned some mechanistic aspects described by the atomic transporter structures [30–32]. The case in point here being a periplasmic substrate binding site in the sodium coupled leucine transporter LeuT, which appeared to be obscured by detergent from the experimental conditions in the structures, leading to two opposing lines of thought regarding its existence, reviewed in [33]. In the light of these evidences, it has become necessary to recall that the transport process takes place in the membrane and to insist on an experimental consideration of the membrane properties in the mechanistic description. This path was already pursued exemplarily for channels, e.g., for the mechanosensitive channels [34]. Structural studies in the presence of native lipids are required to obtain a more holistic understanding of the transport mechanism. Bioinformatics and molecular dynamic simulations of proteins embedded in an artificial bilayer template, which provide the possibility to validate kinetic aspects deduced from structures, will further improve the existing picture [35].

But will it be possible to come up with one unifying molecular description of secondary transport once we include the membrane into consideration? The question emerges on how general such a "membrane-placed" transport description will be, bearing in mind that different organisms have different sets of membrane lipids. Membranes of mammalian cells contain mainly phosphatidylcholine (PC) and phosphatidylserine (PS), while the main component of most bacteria is phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and cardiolipin (CL). The spacious cardiolipin, a di-phosphatidylglycerol, is also an important component of the inner mitochondrial membrane, where it constitutes about 20% of the total lipid composition and plays diverse functional and structural roles (see Section 3.1). In addition minor components such as cholesterol and phosphatidylinositol can play important functional roles forming micro-domains in which proteins are concentrated (see Section 2.1). The differences in head group nature result in a variation of charge and spatial occupation within the membrane that will affect the interaction of lipids with the charged residues of proteins. The generally accepted view in structural biology that the same fold most likely implies similar functional mechanisms suggests that homologous transporters from different kingdoms of life will indeed show a comparable mechanism, although variations in mechanistic details would certainly be accommodated depending on the nature of the transported substrates themselves and specific functions. By additionally bringing the heterogeneity in biological lipid bilayers into the picture, a few imperative hypotheses concerning lipid–protein interactions in transporters can be inferred, for example 1) a co-evolution of membrane composition and transporter protein sequence, 2) the conservation of the position of a lipid-binding site, but perhaps not the exact same coordination, and 3) an increasing role of physical properties of the bulk membrane lipids that might be achieved also by very different lipid compositions. The existing knowledge of functional and structural lipid–protein interactions in transporters proposes some intriguing answers to the aforementioned hypotheses. For instance, the causal effects of lipid–protein interactions observed in the structure of the betaine transporter BetP (Fig. 1) appear to be conserved across one of the most abundant secondary transporter folds,

the leucine transporter (LeuT) like protein fold [36–38], shared by many functionally distant secondary transporters (see Section 4).

2.3. Secondary transporter function is modulated and regulated by lipids

Secondary transporters are active transporters; they exploit the electrochemical membrane potential to energize the movement of substrates across the membrane. More precisely moving of substrates is coupled to the free energy of the electrochemical potential of one substrate to drive uphill transport of another substrate. In the case of a proton or sodium ion gradient the driving potential is denominated as proton-motif-force (pmf) and sodium-motif-force (smf), respectively. The fluxes of substrates are so strictly coupled that neither of them can be transported alone. The electrochemical membrane potential, hence the membrane composition and physical state of the membrane is an important determinant in secondary transport, making them an interesting target to discuss functional lipid–protein interactions.

The intriguing mechanism, by which coupling in secondary transporters is achieved is described by the "alternating-access-model". According to this model, the transporter will bind its substrate(s) to a central binding site, which is only accessible from one side of the membrane at once. For substrate release, the transporter has to undergo substantial conformational changes that first occlude the central site before opening it up again from the opposite side of the membrane. To do so, the transporter has to adopt a sequence of distinct states, which comprise at least an outward-facing conformation (central binding site is accessible from the outward, but occluded from the inward) and an inward-facing conformation (central binding site is accessible from the inward, but occluded from the outward).

2.3.1. Modulation of transport by hydrophobic mismatch

A detailed review of primary and secondary transporters for which lipid–protein interactions have been demonstrated to modulate function can be found in [8]. In a majority of transporters discussed in this detailed review the molecular mechanisms by which lipids exert their influence on these proteins are as yet unclear. Basically, secondary transporters catalyze entry and exit of molecules through the membrane by forming temporary cavities. The formation of the substrate-binding site within the "cavity" might have consequences on both protein and membrane via the so-called "hydrophobic mismatch" [39,40]. The thickness of the transmembrane domains of a membrane protein has to match the thickness of the hydrophobic core of the lipid bilayer, otherwise the contact between hydrophobic and hydrophilic parts of bilayer and protein would cause an enormous energy cost. When substrate binding in a membrane transporter affects the thickness of the hydrophobic protein core, the membrane, to avoid an energetically costly hydrophobic mismatch, will act on the transporter to change its conformation accordingly. Therefore, the transport process itself is most likely modulated — but not necessarily regulated — by the properties of the bulk lipids. This modulation will most likely be different to that reported for channels, because transporters, different to channels, will at no stage during substrate translocation create a continuous pore through the membrane.

The concept of sampling many conformationally distinct states during alternating access implies a structural plasticity of secondary transporters and raises the question of whether specific lipid binding might affect such plasticity by coping with the hydrophobic mismatch [39]. The dynamic changes within the protein will affect the membrane–protein interface that might in turn have an effect on transport [41]. One impressive demonstration in that direction is revealed by the conformational changes observed in the leucine transporter LeuT [42,43], a member of the medically important neurotransmitter:sodium:symporter family (NSS). LeuT was crystallized in the two main conformations of the alternating-access cycle — open to the outside to bind the substrate and open to the inside to release it. A dramatic conformational change from outward to inward is a hinge like movement of the internal segment of transmembrane helix (TMH) 1 (Fig. 2a), which bends around an extended stretch approximately in the middle of the membrane to open the internal pathway and release the substrates. This movement places TMH1 in the middle of the membrane and driven by the hydrophobic mismatch might cause in turn a conformational change that will close the pathway, when the transporter isomerizes back to the outward-facing state [35].

The consequence of hydrophobic mismatch was also elucidated experimentally for the melibiose transporter MelB from *Escherichia coli* [39]. On investigating lipid acyl chain length influence on the activity of the carrier reconstituted into proteoliposomes, it was found that the highest protein activity was only possible when the hydrophobic matching was optimum. Transport of melibiose by MelB was modulated by the surrounding lipids and strongly impaired when the acyl chain lengths did not match that of the protein transmembrane domain, demonstrating that function in transporters is closely linked with the features of the surrounding lipid environment.

2.3.2. Transport regulation by lipid–protein interactions

A large variety of secondary transporters depend on a defined lipid environment, in the sense that they exhibit a localization dependent activity profile [44]. This behavior was described for most mammalian transporters, e.g., for SLC6 transporters [45,46]. Membrane trafficking, insertion and internalization happens in different membranes however, it is difficult to connect these complex processes entirely and directly to lipids. On the other hand, there are transporters that indeed show a direct lipid dependent regulation of transport activity. For instance, the mammalian Na^+/H^+ exchanger NHE3 that regulates salt and water absorption and maintains systemic pH within the physiological range [47] undergoes an electrostatic interaction of the cationic cytoplasmic tail with anionic lipids at the inner leaflet of the plasma membrane that alters NHE3 activity. An additional aspect of regulation of apical NHE3 is the association of NHE3 with cholesterol and sphingolipid-enriched membrane domains (see Section 2.1).

Another prime example for direct lipid-dependent activity regulation is the bacterial betaine transporter BetP, a member of the BCCT (betaine–choline–carnitine-transporter) family. Its physiological function is to accumulate the compatible solute betaine to high concentrations in the cytoplasm in response to hyperosmotic conditions in the cell's surroundings [48,49], which is a common type of stress for bacteria. When the extent of stress exceeds a given threshold, BetP is instantly activated and starts to import betaine into the cell. It is mainly the direct cellular consequences following the change in the osmotic gradient between the exterior and the interior of the cell, e.g., an increase in internal K^+ concentration that can be sensed by BetP via its C-terminal domain leading to activation. Interestingly, the extent of functional change in the regulatory behavior as a result of alterations in the C-terminal domain critically depends on the nature of the lipid surrounding BetP. Moreover, BetP responds to an as yet uncharacterized stimulus arising from the cell membrane in addition to being activated by increasing cytoplasmic potassium ion concentrations during hyperosmotic stress [50]. BetP is specifically activated by this membrane stimulus when the bacteria encounter chill stress, during which there is no significant change in K^+ concentrations, eliminating this stimulus [51]. It was assumed that the transporter is able to sense collective changes in the state of the membrane under such conditions [51].

BetP shows some similarities regarding the C-terminal interactions via arginine clusters with the membrane surface (Fig. 2c) to the regulation mechanism described for NHE3 [52], as its regulation depends on the presence of negatively charged lipids, too [53]. Taken together, the influence of phospholipids on BetP function was found to be twofold. Besides the striking dependence of BetP on the composition of the surrounding membrane lipids, BetP directly depends on the surface charge of the surrounding phospholipid bilayer [54].

At first glance, these two prototypical transporters show an activity regulation due to lipid–protein interactions of structural elements,

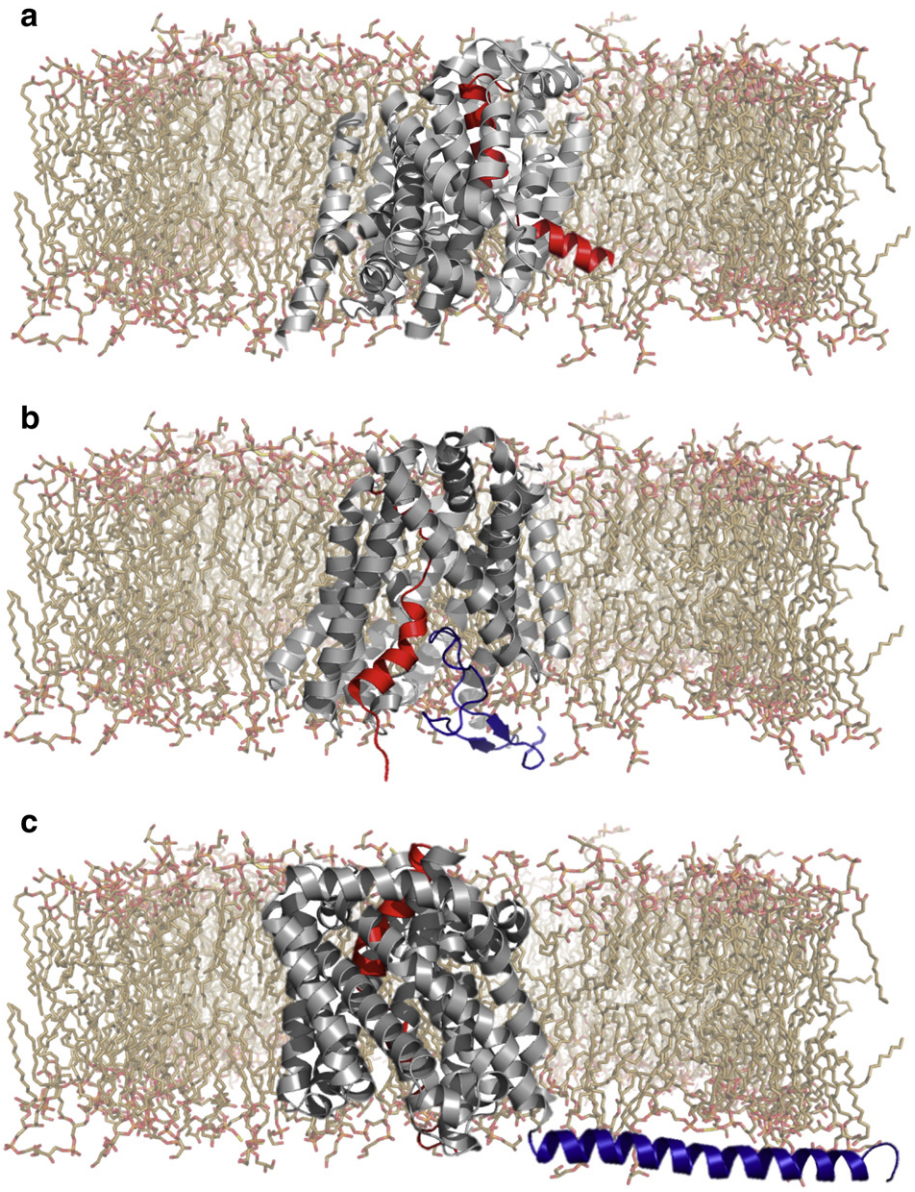

Fig. 2. Conformational changes during the isomerization of the leucine transporter LeuT from outward to inward placing the internal segment of transmembrane helix 1 (in red) deep into the hydrophobic core of a model membrane (PDB: 3TT3, [34]). b) and c) Putative competitive interactions of terminal domains with bulk lipids and the transporter core, respectively, in the LeuT-like fold transporters GadC (b, PDB: 4DJI, [55]) and BetP (c, PDB: 4C7R, [54]). TMH1 is colored in red as in panel a, however, the transporters are shown from different orientations in panels a, b, and c.

which are at the best only membrane associated and do not, technically speaking, belong to the transport unit. These interactions with the membrane surface are of electrostatic nature, which can be altered by environmental changes, e.g., osmotic stress or pH. In BetP regulation, an increased internal K^+ concentration results in a cooperative activity increase suggesting that several cytoplasmic K^+ binding sites are required to alter the nature and/or strength of these interactions.

Recent structural data on the betaine transporter BetP [55], the GABA/glutamate antiporter GadC [56] and the dopamine transporter DAT [57] have shown that terminal interactions with the membrane surface indeed compete with interactions within the hydrophilic parts of the transporter (Fig. 2b, c). Based on these results, regulation by the membrane appears as a part of a partner-switching mechanism of the soluble terminal domains. In that sense the physical properties of the membrane (surface charge, membrane tension and curvature) are important determinants of activity regulation and as a matter of fact those can hardly be observed in a crystalline environment, neither in 3D crystals nor in 2D crystals, in which protein–protein crystal contacts will not inhibit specific lipid–protein interaction, but distort the bulk lipid properties significantly.

3. Impact of lipid interactions structurally observed in transporters

Despite the limitations of resolution and inherent flexibility of lipid–protein interactions, few transporter structures have revealed bound lipids (Table 1). Fragments or whole lipid and detergent molecules have been reported at a range of resolutions from 1.8 to 3.9 Å in transporter structures (Table 1). Most structures, for which bound lipids were observed, were obtained by vapor diffusion methods. However, no distinct trend can yet be identified since new structures with lipid cubic phase methods are steadily emerging. Interestingly, native lipid fragments have been identified in a majority of these structures with bound lipids (Table 1), implying their underlying functional significance. Most of these structurally resolved lipids are also observed on the interior half these transporters which are rich in positively charged residues in keeping with the positive-inside rule for transmembrane

Table 1
Crystal structures of transporters with resolved lipid/detergent molecules. Known contributions of these molecules are classified as structural when they influence crystallographic or structural stability and/oligomerization, and functional for direct effects. (The reader is directed to [8] for a listing of phospholipid compositions of selected membranes.)

Protein	Organisms		Native plasma membrane content	Crystallization method	Res(Å)	Lipid/detergent species resolved	PDB code	Contribution/ reference
	Native	Host						
Dopamine transporter	*Drosophila melanogaster*	Virus infected mammalian cells	SM, CHL, CHLester, CL, PE, PC, LPC, PI, PS, TAG [84]	Vapor diffusion	2.95	CHL	4M48	Functional[75]
Betaine transporter	*Corynebacterium glutamicum*	*E. coli*	PG, CL, PI[53]	Vapor diffusion	2.7, 3.3	PG	4C7R, 4DOJ	Structural and functional [55]
Carnitine-G butyrobetaine antiporter	*Escherichia coli*	*E. coli*	PE, PG, CL [85]	Vapor diffusion	2.29	Cymal 5	2WSW	Structural [86]
Leucine transporter	*Aquifex aeolicus*	*E. coli*	Diverse: Ester, mono- and di-ether lipids[a], APT lipids [87] among reported	Vapor diffusion or lipid bicelle	1.85, 2.0, 2.9, 2.0, 2.25, 2.63, 3.1, 2.5	β-OG or S-OG or PC head group	2QEI, 3F3A, 2QJU, 3GJD, 3MPN, 3QS4, 3TT1, 3USG	Structural[42,69] Functional [88][d]
Aspartate transporter	*Pyrococcus horikoshii*	*E. coli*	Caldarcheol and archaeol lipids[b]	Vapor diffusion	2.96	Palmitic acid acyl chain fragment	2NWL	n.d./[89]
ADP/ATP carrier	Bovine heart mitochondria	Bovine heart mitochondria	SM, CHL, CL, PE, PC, PI, PS[c] [90]	Vapor diffusion	2.2, 2.8	CL, PC, LDM	1OKC, 2C3E	Structural and functional [61]
Bile acid transporter	*Neisseria meningitidis*	*E. coli*	PE, PG, PA, CL [91]	Vapor diffusion	2.2	PE, LDA	3ZUY	n.d./[74]
	Yersinia frederiksenii	*E. coli*	n.d.	LCP	1.9 2.5	MPG	4NYW 4NYX	n.d./[92]
Maltose transporter	*Escherichia coli*	*E. coli*	PE, PG, CL [85]	Vapor diffusion	3.1, 2.2, 3.91, 2.9	PG	3PV0, 3RLF, 4JBW, 4KHZ	n.d. [93]
Human ATP binding cassette, ABCB10	Human	Insect cells using baculovirus vectors	SM, CHL, CL, PE, PC, PI, PS[b] [90]	Vapor diffusion	2.85	CL	4AYT	Structural [94]
FucP, MFS transporter	*Escherichia coli*	*E. coli*	PE, PG, CL [85]	Vapor diffusion	3.14	β-NG	3O7Q	n.d./[95]
Sodium/calcium exchanger NCX	*Methanococcus jannaschii*	*E. coli*	Archaeal lipids [96]	Lipid cubic phase and vapor diffusion	1.9	1-Oleoyl-R-glycerol	3V5U	n.d./[97]
Concentrative nucleoside transporter	*Vibrio cholerae*	*E. coli*	n.d.	Microbatch under oil	2.44	DM	3TIJ	n.d./[98]
UraA uracil/H+ symporter	*Escherichia coli*	*E. coli*	PE, PG, CL [85]	Vapor diffusion	2.78	β-NG	3QE7	n.d./[99]
AdiC arginine:agmatine antiporter	*Escherichia coli*	*E. coli*	PE, PG, CL [85]	Vapor diffusion	3.0	β-NG	3L1L	n.d./[68]
Thiamin transporter	*Lactococcus lactis*	*L. lactis*	PG, CL, glycolipids and glycerophosphoglycolipids[e]	Vapor diffusion	2.0	β-NG	3RLB	n.d./[100]

n.d.—not determined.
SM—sphingomyelin, CL—cardiolipin, TAG—triacylglycerol, PG—phosphatidylglycerol, PE—phosphatidylethanolamine, PI—phosphatidylinositol, PS—phosphatidylserine, CHL—cholesterol, APT—phosphoaminopentanetetrol, PC—phosphatidylcholine, LPC—lysophosphatidylcholine, β-OG—β-octylglucoside, S-OG—S-octylglucoside, β-NG—β-nonylglucoside, DM—decyl-β-d-maltopyranoside, LDA—lauryl dimethylamine-N-oxide, LDM—3-laurylamido-N,N′-dimethylpropylaminoyde, MPG—1-monooleoyl-rac-glycerol.
[a] Source: http://arxiv.org/pdf/1309.4467.pdf.
[b] Source: Journal of Japan Oil Chemists' Society; ISSN: 1341-8327; Vol. 49; No. 7; (2000).
[c] Lipid content in plasma membrane.
[d] OG is bound to the second substrate site in LeuT, establishing an inhibitor-bound conformation.
[e] Source: http://dissertations.ub.rug.nl/faculties/science/1992/g.e.in.t.veld/.

protein topology determination [58] and serve to coordinate negatively charged lipid head groups. These structures are now providing early insights into how transporters use lipids to mediate intra-protomer contacts and possibly cross talk. They also indicate a role for bilayer lipids in stabilizing distinct conformational states in the functional cycle of transporters, as will be detailed in following sections.

A common feature of lipids resolved in structures of channels and receptors is to mediate oligomerization [8,18,59]. Lipids are frequently found to establish contacts between monomers and also plug spaces between assembled oligomers, providing an efficient membrane seal [2]. Being restricted between protomers these non-annular lipids are relatively easily resolved and identified in crystal structures compared to the more flexible annular lipids that surround the protein. It is therefore non trivial to distinguish additional functional roles that non-annular lipids may perform in addition to mediating oligomerization. On the other hand, annular lipids when structurally resolved are often found localized around regions of functional importance. We will discuss two examples for structurally described specific lipid–protein interactions in secondary transporters in more detail: the mitochondrial ADP/ATP carrier (AAC) and the osmostress regulated bacterial betaine transporter BetP.

3.1. Cardiolipin binding to the mitochondrial ADP/ATP carrier

The first and groundbreaking example for structurally validated specific lipid–protein interactions is the mitochondrial ADP/ATP carrier (AAC) that interacts with cardiolipin [60]. The ADP/ATP carriers are highly expressed in the mitochondria and facilitate import of ADP and export of ATP into the mitochondria for ATP regeneration. Malfunction of these transporters are implicated in severe diseases like mitochondrial myopathies [61]. Binding of CL, which is the major component of the mitochondrial inner membrane is not astonishing, but the large variety of lipid–protein interactions in the AAC, ranging from a tight specific cofactor type binding to the contribution of CL to the surrounding lipid bulk is remarkable. CL is essential in the stabilization of the carrier during purification and crystallization and also required during transport measurements when reconstituted in proteoliposomes. The crystal structure of AAC (Fig. 3a, b) revealed partially resolved cardiolipin and phosphatidylcholine lipid molecules and detergent molecules. Three cardiolipin molecules remained attached to the crystallized monomer along with four phosphatidylcholine molecules. The functional unit of the carrier is still a matter of debate [62], however the structure reveals a dimer stabilized by the cardiolipin interactions between monomers [63]. The authors also propose that the cardiolipin interface may play a functional role by transmitting conformational changes from one monomer to the other facilitating a concerted ADP/ATP exchange.

AACs exhibit an extraordinarily strong interaction with CL and the stoichiometry of three CL molecules per monomer was also corroborated by NMR and EPR measurement. The unusual binding strength was also reflected by the fact that bound CL did not exchange in the NMR experiment [64]. The tight binding of the negatively charged CL molecules can be rationalized by a collar of positive charges, mainly lysine, at the height of the membrane surface [61]. Aside from the general stabilizing effects of CL on the mitochondrial AAC, CL is also suggested to have a selective effect on transport activity [65], and moreover on substrate coupling within this antiporter [63]. In this context, it was assumed that the tightly bound CL might have a co-factor role for facilitating transport by providing additional electrostatic energy.

3.2. Phosphatidyl glycerol interaction in the betaine symporter BetP

A recent structure of the trimeric BetP in complex with anionic lipids revealed a plug of phosphatidyl glycerol lipids between its three monomers (Figs. 1b and c and 3b). It was proposed that these lipids would provide additional intra-trimeric contacts enhancing the stability of the oligomeric assembly ([55], Fig. 4a, b). Similarly, trimeric carnitine-gamma butyrobetaine antiporter CaiT, also from the betaine choline carnitine transporter (BCCT) family like BetP, presented Cymal-5 detergent molecules bound at equivalent sites in its oligomeric assembly, supporting the possibility of lipids mediating protomer contacts in these transporters.

BetP is known to have a clear dependence on anionic lipids, which are also the major components of its native *Corynebacterium glutamicum* membranes ([55] and Table 1). Transport activity and regulation are severely compromised in cells lacking negatively charged lipids [53]. In osmotic stress response this behavior was also observed in the primary active transporter OpuA indicating a more general theme in stress regulation that is not limited to secondary transporters [66].

Structural resolution of anionic lipids in BetP now provides an opportunity to correlate the known functional modulation by lipids to specific protein–lipid interactions and identify hot spots. Indeed, the authors observe that along with mediating trimer contacts and possibly assisting oligomerization, lipids were resolved at sites that were also previously identified as key elements in the conformational changes BetP undergoes to transition from an outward to inward open state [55]. A lipid-binding site was resolved along TMH1 (Figs. 2c, 5a and 6b, in red) the main cytoplasmic helix gating inward opening in this transporter. An unwound stretch in this helix coordinates substrates and also undergoes large movements during conformational transitions to translocate substrates [67]. The resolved lipid molecule was seen to form van der Waals interactions with a methionine residue in this critical stretch in BetP (Fig. 5a). This interaction was resolved in the inward open state structure of BetP, possibly stabilizing the non-helical stretch

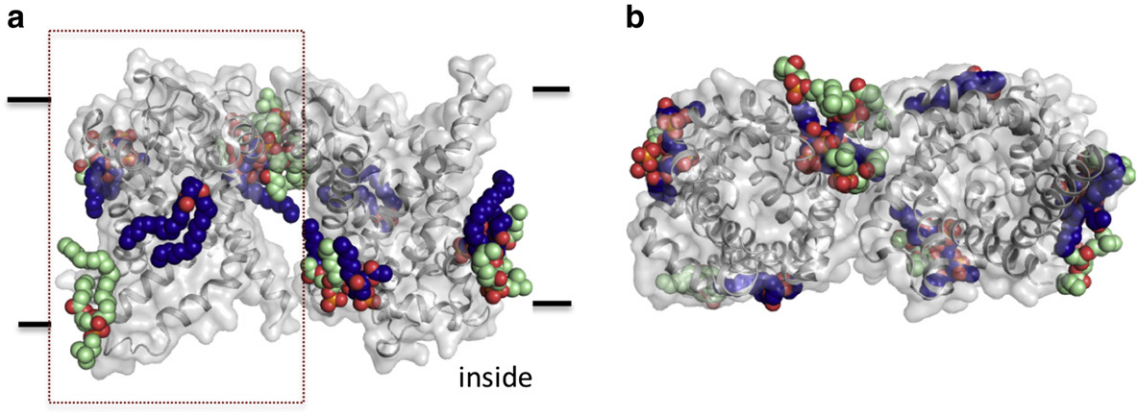

Fig. 3. Crystal structure of the ADP/ATP carrier (PDB: 1OKC), with a symmetry molecule forming the dimeric assembly with cardiolipin molecules at the interface. Cardiolipin molecules are shown as blue spheres and phosphatidyl choline in green, a) membrane view with the symmetry molecule highlighted in a red box, b) top view.

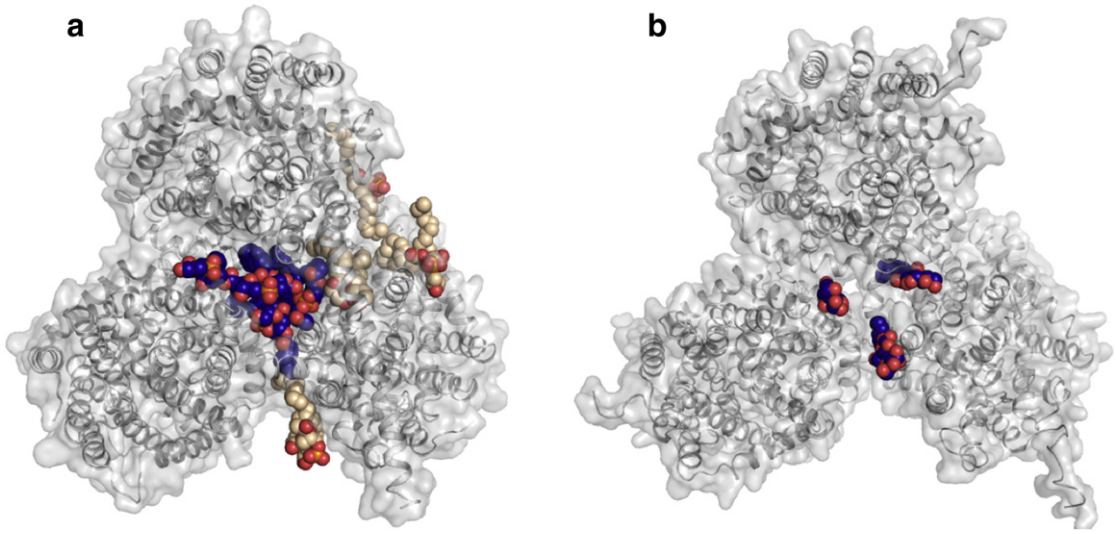

Fig. 4. a) Crystal structure of BetP in complex with lipids (PDB: 4C7R). Non-annular PG lipids are shown in blue and annular lipids are shown as wheat spheres. b) Crystal structure of CaiT (PDB: 2WSW). Cymal 5 molecules between monomers are shown as blue spheres.

in this conformation. While a clear-cut mechanism for bilayer influence on BetP function remains to be adduced, the authors proposed that specific conformations in the functional cycle might be favored by lipid–protein interactions [55].

4. A common theme for functional lipid interactions in the LeuT fold?

BetP adopts the leucine transporter (LeuT)-like fold, which is also shared by many functionally unrelated transporters [37]. Similar TMH1 helix interaction with lipids also seems possible in another LeuT-like fold amino acid antiporter AdiC [68]. A methionine residue on the unwound stretch in TMH1 of AdiC appears potentially exposed to the bilayer from the crystal structure. A sphere representation of this structure in fact shows a groove at this site that may fit a bilayer lipid acyl chain, alluding to the possibility of lipids also stabilizing conformational states in this antiporter (Fig. 5b).

Although no significant lipid interactions were resolved in crystal structures of LeuT, detergent molecules attached to the protein periphery were found in nearly all reported structures (Table 1, [42,69]). MD simulations of LeuT suggested a role for bilayer thinning to compensate for hydrophobic mismatch around its TMH1 helix. Conformational changes associated with transport were identified in LeuT based on different structures of intermediate states [43], based on which TMH1 was seen to navigate large changes during conformational transitions. It was suggested that membrane alterations around this helix would impede movements necessary to open the transporter inwards, possibly stabilizing the outward facing state in LeuT as a consequence [70].

Surprisingly, a recent crystal structure of the dopamine transporter DAT from *Drosophila melanogaster* that was also found to share the LeuT fold, added to this repertoire of examples where the TMH1 helix participates in lipid interactions. This medically important transporter belongs to the neurotransmitter:sodium:symporter (NSS) family of which LeuT is a bacterial homologue [57]. They are involved in the termination of neurotransmission signals at synaptic clefts by driving the

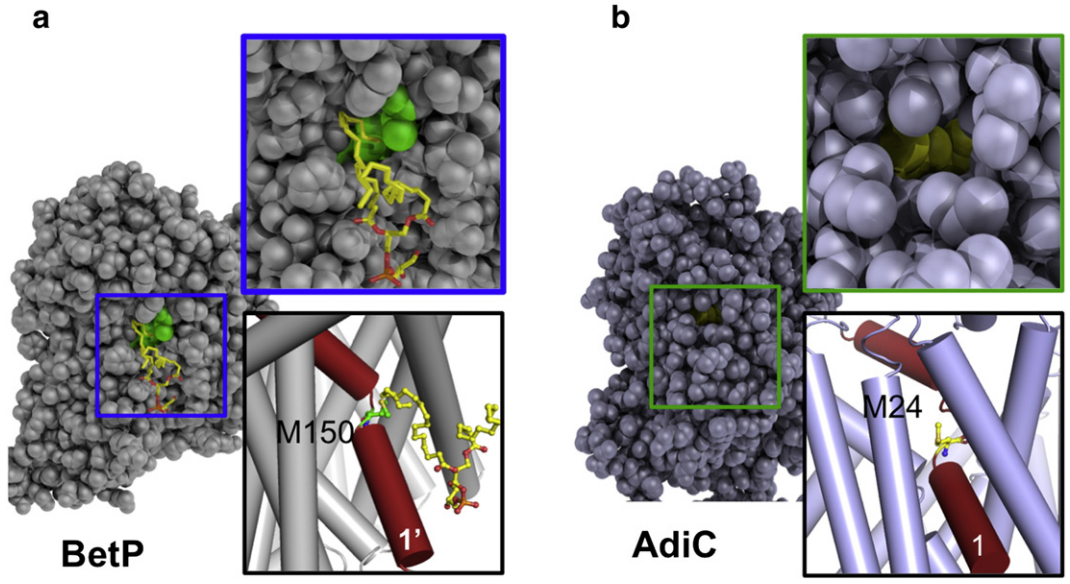

Fig. 5. a) Sphere representation of chain C of the BetP structure (PDB: 4C7R), showing a lipid acyl chain (in yellow) interacting with a hydrophobic groove in the transporter. A methionine residue in the unwound stretch of helix TM1 (numbering adapted to that of LeuT for comparison [55], interacts with the acyl chain. b) Sphere representation of the crystal structure of AdiC (PDB: 3L1L), showing a similar hydrophobic groove as BetP, with a methionine residue in the unwound stretch of TM1 exposed to the bilayer.

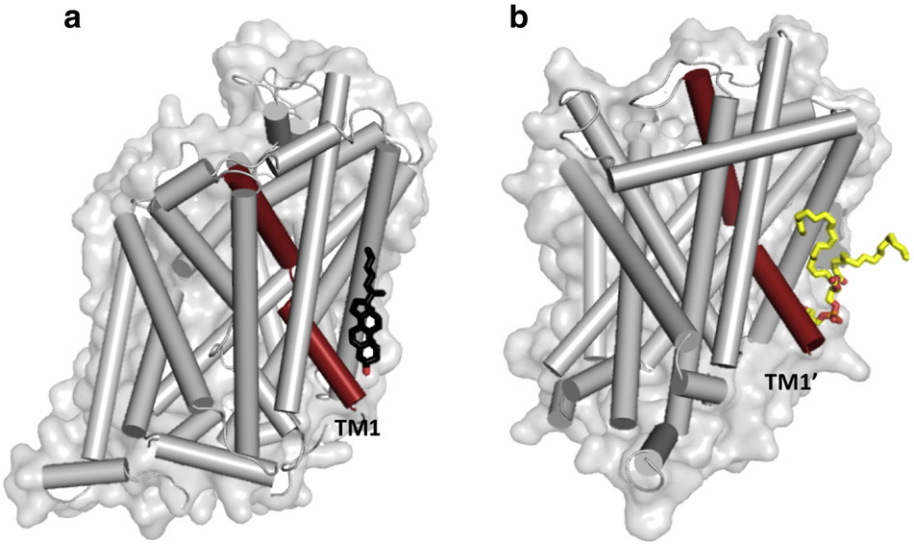

Fig. 6. a) Crystal structure of DAT (PDB: 4M48) in complex with a cholesterol molecule shown in black. TM1 one of the helices lining the cholesterol-binding groove is shown in red. b) A similar lipid-binding site is seen in the structure of BetP (PDB: 4C7R, chain C), where the lipid acyl chain interacts with residues in TMH1.

uphill transport of neurotransmitters. Their malfunction is implicated in several debilitating disorders like epilepsy, depression, Parkinson's disease and the like. These proteins are known to be functionally modulated by plasma membrane cholesterol content [71].

The crystal structure of DAT presented a cholesterol molecule wedged in a groove bordered by the TMH1 helix whose equivalent in the LeuT structure is implicated in conformational changes required to open the transporter inwards in its functional cycle (Fig. 6a). The authors reasoned that cholesterol binding to this site would therefore favor the outward facing state and provide allosteric control of transport [57].

Based on the lipid interactions resolved in BetP and DAT, a common theme of lipids mediating conformational stability appears to be emerging for LeuT-fold transporters. Other transporters of the NSS family are also known to be modulated by cholesterol, like the serotonin transporter (SERT). Recent electrophysiology experiments established that signaling lipid PIP2 was also needed for function in SERT like in inward rectifier potassium channels and a potential PIP2 binding site was identified [72]. Although no structural information is as yet available for SERT, the crystal structure of closely related DAT now sets the platform for homology modeling studies. In this context, the structural cholesterol-binding site from DAT can also be extrapolated to SERT. Knowledge of specific modulating lipid binding sites in these medically relevant proteins will in turn provide deeper insights into their regulation, essential in designing targeted therapeutics.

Membrane lipids are also observed to influence conformations of transporters belonging to other protein folds for e.g.: in the sodium coupled aspartate transporter GltPh belonging to the glutamate transporter superfamily. EPR spin labeled distance measurements of carefully chosen residues within this homotrimeric transporter indicated that the transport domain of the protein samples both outward and inward conformations in detergent micelles, with a higher fraction of protein in the inward facing state upon addition of the coupling ions and substrate. On the other hand different intermediate conformations were detected when the transporter was reconstituted into proteoliposomes and used for EPR measurements, indicating a clear effect of the lipid membrane on the states sampled during transport [73]. Membrane-stabilized protein conformations therefore appear to be a commonly employed strategy for function modulation across unrelated protein folds in transporters.

5. Conclusions

The structural examples detailed in this review reveal the molecular basis of how lipids influence transporters by aiding communication between functional units of transporters, enhancing structural stability and by stabilizing specific conformational states in its functional cycle. Not surprisingly, specific lipid–protein interactions occur dominantly with negatively charged lipids as cardiolipin [63], PG [55] and PIP2 [72] exploiting electrostatic interactions at charged membrane surface, while the hydrophobic interactions are less specific and most likely maintained by exchangeable bulk lipids [74]. The examples described here also demonstrate that transporters utilize both specific lipid protein interactions and the effects of bulk lipid properties to modulate their activity. While specific lipid–protein interactions may stabilize certain mechanistic conformations in transporters, bulk lipid properties could aid in partner-switching mechanisms to activate these proteins. A common pattern of localized lipid binding sites is also seen in structures of two very distant structural homologues, DAT and BetP. Interestingly, neither the exact coordination nor the lipid specie itself is conserved, only the position (Fig. 6) reflecting how membrane lipid composition and transporter sequence have co-evolved. These molecular details lay the foundation for the design of further structure based mutagenesis approaches, e.g., thermostabilization [75,76] and theoretical MD simulations, to understand functional mechanisms of transporters in a more complete manner. Keeping in mind the importance of resolving membrane interactions with proteins, new methods that include new detergents, e.g., amphiphilic MNG species [77], native lipids or adding lipids known to modulate function into the crystallization experiment are increasingly being used [78]. Lipid cubic phase or bicelle crystallization methods provide membrane-like environments that stabilize fragile membrane proteins or help to maintain native conformations of regions which depend on bilayer lipid interactions [16]. Along with strategies to maintain lipid interactions in crystals, it may also be useful to carefully interpret available data for any density matching that of lipids/detergents. Although this is only possible on a case-by-case basis where probable lipid-like densities are observed, iterative model building and refinement after including these species may improve identification and provide potentially useful lipid–protein interaction information. The annular lipids resolved in BetP (PDB: 4C7R) for example show different degrees of flexibility and several elongated densities were observed in the structure. Initial placement of lipids moieties within these densities improved phases and connections with lipid head groups could be traced after iterative cycles of model building and refinement to arrive at the best lipid position from the observed densities (Fig. 7).

Finally, the question remains if crystallization will be the method of choice to describe lipid–protein interactions in molecular detail, especially

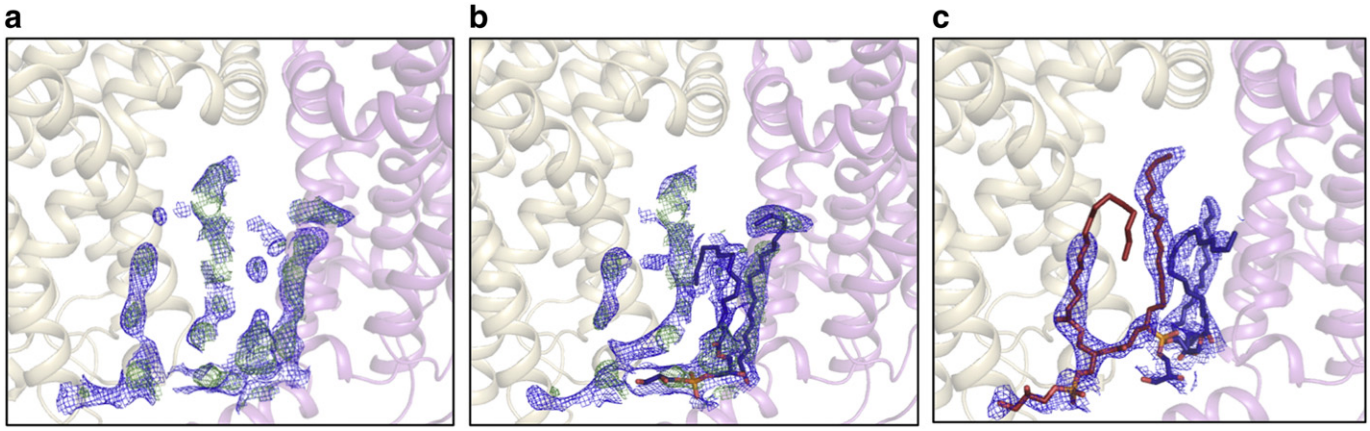

Fig. 7. Progressive improvement in phases around potential lipid binding sites during structure refinement in BetP: Annular lipids when present are usually flexible and difficult to interpret. An example of the refinement of two such lipid densities in BetP is shown, where panels a and b depict initial rounds of model building and refinement. 2Fo–Fc densities (blue) are contoured at 0.6σ and Fo–Fc densities at 2.5σ. Features could be identified as phases improved with iterative model building and refinement and clear connections between head group and acyl chains were visible towards the end of refinement (panel c, 2Fo–Fc at 0.8σ).

since most biological membranes exist in a disordered, fully hydrated state. To elucidate their fine structure neutron and X-ray scattering methods as well specialized NMR studies were recently proposed [79]. In addition, the investigation of fine membrane structures requires stronger emphasis on pushing forward light microscopic techniques [80], spectroscopic force measurements by AFM [81], single molecule techniques [82] and single particle electron microscopy [83] for transporters to complete the molecular mechanism.

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