Substrate Trajectory Through

Phospholipid Transporting P4-ATPases

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Synopsis:

A difference in the lipid composition between the two leaflets of the same membrane is a relatively simple instance of lipid compositional heterogeneity.. The large activation energy barrier for transbilayer movement for some (but not all) membrane lipids creates a regime governed by active transport processes. An early step in eukaryote evolution was the development of a capacity for generating transbilayer compositional heterogeneity far from equilibrium by directly tapping energy from the ATP pool. The mechanism of the P-type ATPases that create lipid asymmetry is well understood in terms of ATP hydrolysis, but the trajectory taken by the phospholipid substrate through the enzyme is a matter of current active research. There are currently three different models for this trajectory, all with support by mutation/activity measurements and analogies with known atomic structures.

Keywords: Phospholipid, flippase, P4-ATPase, giant substrate

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Introduction:

Biological membranes separate cytoplasm from the extracellular space or the lumens of intracellular organelles because they present a barrier to the diffusion of ions and other hydrophilic molecules. Among the molecules that experience this barrier are the phospholipids that form the bilayer itself. Because each leaflet of the bilayer must occupy the same surface area while the enzymes responsible for membrane biogenesis are cytoplasmic, organisms evolved mechanisms to facilitate rapid equilibration of phospholipids between the two leaflets [1, 2], . The appearance of eukaryotic cells and their internal organelles separated such biogenic membranes (the ER and mitochondria) from the plasma membrane and associated compartments (endosomes, sorting organelles, etc) where this equilibration does not occur. At the same time, membrane transporters appeared that organize the movement of phospholipids between leaflets, including molecules (scramblases) that can activate rapid equilibration [3] and molecules (translocases or flippases) that use ATP to create lipid asymmetry, i.e., a non-random distributions of phospholipids between the two leaflets [4].

The identity of these molecules are only just coming to light in the case of the scramblases [5-8], and the structural requirements for this activity may be quite permissive [9, 10]. On the other hand, the molecules that actively transport phospholipids have been known for some time [11] – they are Type IV P-type (or P4) ATPases [12]. Because they are P-type ATPases, much is already known about their structure and molecular mechanism [13]. The P4-ATPases have the same basic sequence organization (10 transmembrane domains, with two large cytoplasmic loops between TM2 (TransMembrane helix 2) and TM3 and between TM4 and TM5) and thus the same basic structure, as the Ca- [14], NaK- [15], and proton [16] ATPases. Unlike most of the other P-type ATPases, the P4 ATPases are associated with a subunit from the Cdc50 family of membrane proteins [17] required for their activity [18, 19].

P4-ATPase reaction cycle:

The P-type ATPases operate by the Post-Albers cycle [20, 21] between a cytoplasmic-facing E1 conformation and a lumenal-facing E2 conformation. Phosphorylation of a conserved aspartate in the canonical sequence DKTGTLT drives a switch from E1 to E2; this aspartate is present and phosphorylated in the P4 ATPases. Conversion of E2 back to E1 is driven by hydrolysis of the aspartyl phosphate catalyzed by a conserved glutamate in the first cytoplasmic domain; mutation of the corresponding glutamate in the P4 ATPases is sufficient to block transport [18]. Together, these experiments show that the basic ATP hydrolysis cycle in the P4 ATPases is the normal sequence studied in detail for the Ca- and Na-K ATPases [13, 22].

While these insights are both helpful and important, the demonstration that phospholipids are transported by the P4 ATPases [23, 24] posed an obvious biochemical problem which cannot be

addressed by reference to other P-type ATPases. We know that there is a specific binding site for the phospholipid, and we know that the site is not compatible with sphingolipids, that it can accommodate some, but not all, headgroups, and that it is stereospecific for the central carbon of the glycerol backbone [25]. Understanding the molecular basis of these specificities is not in prospect, because it is not clear how the phospholipid molecule passes from one side of the membrane to the other. This puzzle is generally described as the "giant substrate" problem, but while size is one important component, it is not the only one. While phospholipids are much larger than the ions transported by most P-type ATPases, they have at least two other properties which must be accommodated by the transporter. One is that phospholipids are amphipathic, so that the passageway through the membrane must handle a substantial hydrophilic headgroup while not inconveniencing the attached fatty acid side chains. The second is that the phospholipid molecule is asymmetric, which means that the transport process must include a reorientation of the molecule. The location of the various moving parts, and of the residues which control the specificity of transport, are questions that are in principle susceptible to analysis by the kind of site-directed mutagenesis that illuminated the ion binding sites and critical residues in the other members of the P-type ATPase family, if an assay for the effect of those mutations is available.

There are three basic assays which have been used to analyze how phospholipid transport by P4-ATPases, each with their own weakness. One is reconstitution of purified protein into artificial vesicles, followed by measurement of the ATP-dependent changes in the distribution of phospholipid probes incorporated into the vesicles [26, 27] . This approach is conceptually attractive, but technically limited. Transported probe is part of the bilayer, which is easily destabilized if transfer of phospholipid from one leaflet to the other creates too large a difference in the area of the two leaflets. Moreover, the small size of vesicles combined with appropriate limitation of the amount of transported probe means that the reaction should reach a plateau in a few seconds, making kinetic measurements impossible. A second method is measurement of ATPase activity in detergent-solubilized enzyme [26, 27]. This method is technically straightforward, but doesn't measure transport itself, and these enzymes can catalyse ATP hydrolysis in the absence of measurable transport. In addition, in these assays substrate is presented in detergent-solubilized form, making it difficult to interpret effective substrate concentrations. The third assay is to measure internalization of fluorescent phospholipid analogues in living cells [28, 29]. This assay permits kinetic measurements and can be used to make measurements of substrate affinity [29], but it has the problem that the effects of cellular factors including regulation and cellular distribution may be indistinguishable from effects on enzyme activity itself. Finally, there are several assays such as cold-sensitivity of growth [30], binding of antibiotics [31, 32], and localization of endogenously expressed lipid probes [32], that do not measure enzyme activity at all, but may be related to it and are sometimes used in conjunction with other, more direct assays.

All of these assays have been deployed to try to define the path taken by phospholipids through the transporter; three distinguishable pathways have been suggested, each with strengths. In all cases, the proposed pathway is derived from features of ion transport by other P-type ATPases. These pathways are discussed here in turn.

Two-gate model:

The first, and oldest, proposal for the pathway is that the phospholipid passes along the surface of the transporter [33]. The first version of this proposal was provoked by the structure of the E2 form of the Ca-ATPase (2AGV, [34]), which has a phospholipid lodged in a cleft between transmembrane helices 2 and 4 on the cytoplasmic side of the membrane. Remarkably, the binding site for this phospholipid depends primarily on interactions with the glycerol backbone, suggesting that it has the stereospecificity characteristic of the P4 ATPases (above). This phospholipid is ejected at the E2 to E1 transition, and if it is replaced later in the reaction cycle by a phospholipid from the lumenal side of the bilayer, the result would be phospholipid transport [33]. Experimental support for this site came from domain swaps between two yeast P4 ATPases, as assessed by probe transport by living yeast [28]. A relatively conservative substitution of Phe (the phosphatidylserine (PS)-transporting Drs2 residue) for Tyr618 in (the phosphatidylcholine (PC) transporting) Dnf1 increased the ratio of PS to PC transport. This tyrosine is on the cytoplasmic side of the membrane, and corresponds to a proline that is part of the phospholipid binding site in the 2AGV structure. Together, these data suggested that a binding site, specific for phospholipid headgroup, is present at the surface of Dnf1. Curiously, selection for resistance to a toxic PC analog identified F587, a residue in a loop between TM3 and TM4 on the lumenal side of the membrane. Based on these observations, the authors suggested that phospholipid passes through the membrane along the surface cleft between TM1, TM3, and TM4, with a specificity-determining site at the cytoplasmic surface [28].

These results were extended using similar methodology to the loop between TM1 and TM2 [35], where swapping Drs2 amino acids into Dnf1 and measuring relative transport of PC and PS led to proposals that amino acids in this region on the lumenal side of the membrane also controlled both PS and PC specificity. Selection of randomly mutagenized yeast for resistance to a toxic PC analog gave rise to strains with mutations in TM3, including two near the cytosolic interface that were proposed to influence specificity by the same criteria used to interpret the TM1-TM2 loop mutations. Curiously, substitution of a variety of amino acids for one residue (N550) increased the apparent specificity for PS, even though this residue is also an asparagine (N445) in the PS-transporting Drs2. The same assay also uncovered mutations in the TM3 and TM4 lumenal loop, in TM4 at the cytoplasmic interface, and in TM6 (although the last was dismissed as an alteration of helix packing). Further investigation of the N550 mutation and mutants identified in a screen using random mutagenesis of Dnf1 TM1 and TM2 and selection

for complementation of the cold-sensitive growth phenotype of Drs2 deletions led to identification of several more mutations (F213S, T254A, and D258E) with apparent increase in PS specificity [32], one of which (T254) corresponds to a residue quite close the phospholipid in the 2AGV structure.

Altogether, these results (Figure 1A) have been interpreted as support for a "two-gate" model for the P4 ATpases [35]; a clear implication seems to be that the two "gates" are barriers without being binding sites. The phospholipid is assumed to progress along the surface of the transporter, while the fatty acid side chains remain imbedded in the bilayer core, thus solving the problem of how the enzyme accommodates the bulky hydrophobic portion of the phospholipid. The model incorporates the known specific phospholipid binding site in the 2AGV structure, although the model seems to reverse the orientation of the phospholipid, so that the fatty acid tails protrude from the opposite side of the transporter than that seen in the Ca ATPase structure. One difficulty with the model is just the number and dispersion of mutations which seem to affect specificity;. On the one hand, this is consistent with the observation that the specificity of the P4 ATPases for headgroups is not stereospecific, and hence may not involve specific interactions with the protein. On the other hand, the authors conflate headgroup and backbone specificity on some occasions, and also propose that "binding" to the cytosolic "gate" is a key to enzyme progression through the reaction cycle [35], a function which would seem to require specific protein-substrate interactions.

Ca path model:

A second, and completely different, model has been suggested by studies of the mammalian ATP8A2 transporter, studied in vitro in detergent and in reconstituted vesicles. The first evidence for this model came from mutagenesis of a conserved lysine (K873 in the bovine enzyme). The activity of this mutant enzyme was low, and the apparent Km of the enzyme for PS, measured by stimulation of ATPase activity in the presence of detergent, was much lower in the mutant than in the wild-type enzyme. This residue is homologous to S767 in the Ca-ATPase, a residue involved in forming the binding site for one of the two Ca ions in the E1 structure of the enzyme (1SU4 [14]). This result provoked a model in which the phospholipid takes a pathway through the enzyme that is internal, and corresponds to the path taken by Ca ions in their journey through the Ca-ATPase. Much more extensive and detailed support for this model was provided by mutagenesis studies of the same enzyme, including extensive mutagenesis of the isoleucine in the canonical PISL sequence [27]. As was noted when the P4 ATPase subfamily was first identified [11], this isoleucine corresponds to the glutamic acid in the conserved PEGL sequence, a glutamate that is an important part of the binding site for Ca in the Ca-ATPases. Replacement of this isoleucine with any of several uncharged amino acids results in dramatic inhibition of enzyme ATPase activity, with substantial changes in apparent

Km for PS and PE (but not always in parallel). Similar phenotypes are observed upon mutation of several other hydrophobic amino acids in TM1 and TM2. From these observations (Figure 1B), a "single gate" model was developed, with a pair of internal water-filled channels leading to a hydrophobic cluster in the center of the bilayer, roughly in the position of the ion binding sites in Ca- or the Na/K-ATPase [27].

The strength of this model is its minimal departure from what is known about the transport mechanism for substrates of other P-type ATPases. The internal water-filled channels have known precedents, and a threaded structure based on the E2P and E2 structures of the Ca-ATPase was developed that illustrated this aspect of the proposed mechanism. Although the disposition of the fatty acid side chains during passage is not so obviously solved in this model, there is an available slot between TM2 and TM6 which would allow these groups to remain associated with the bilayer core. The most obvious weakness of the model is that it makes no provision for a binding site for the phospholipid headgroup, which is a hydrophilic, not a hydrophobic, entity. But a binding site must exist, both to account for the substrate specificity of P4-ATPase mediated transport, and to account for the regulation of the dephosphorylation step that is the transition between the E2P and E2 forms of the enzyme.

Proton pump model:

One final proposed model is related to the model based on the Ca- and Na/K-ATPases, but is based on the known structure of the proton pump [16]. The starting point for this model is the K873 mutant described above [26]. As noted, the corresponding residue, Ser797, in the Ca-ATPase contributes to the binding pocket for the transported Ca ions, but the interacting atom in Ser797 is the backbone oxygen, while the side chain of Lys873 is important for the P-type ATPase, and it points to the space between TM3/4 and TMs 5/6, the location of a water-filled cavity in the center of the proton pump. A second point came from characterization of the phenotypes of mutations in the yeast enzyme Dnf2 corresponding to disease-causing alleles in the mammalian enzyme ATP8B1/FIC1 [29]. One of these mutations (N601F) , which causes a relatively mild form of the disease when it occurs as I334F in ATP8B1, displayed a relatively minor diminution of activity, but an increase in the apparent Km for external phospholipid, suggesting that it contributes directly to substrate binding. This residue on TM3 is at roughly the same level in the bilayer as the Y618 residue in ATP8A2, and its sidechain also faces in the direction of the space between TM3/4 and TM 5/6

There are several reasons for considering the proton pump cavity as a model for thinking about phospholipid movement. The simplest one is size: at the point of recognition and binding, the transporters must accommodate a hydrophilic headgroup , glycerol backbone, and ester linkages with a volume of over 320 A^3 [36]. While not all of those atoms will be involved in specific linkages, they still occupy a space which is alternately exposed to the two sides of the

membrane as the reaction cycle proceeds. The water filled cavity in the proton pump is about the size of the phospholipid headgroup (about 380 A^3), implying that a cavity of about that size could house the binding site for the phospholipid. At the lumenal end of the proton pump, the structure is more open, and another PFIC related mutant converts a highly conserved glutamate to lysine in the loop that links TM5 and TM6 in this region. In ATP8B1, this mutation results in severe disease, and the corresponding mutation in the yeast Dnf2 enzyme (E1261K) has a severe defect in transport rate, with a substantial decrease in the Km for PC [29]. Given the position of the mutation, these results argue that mutant lysine creates a binding site on the lumenal surface at an entry point which can lead to an internal binding site between TM3/4 and TM5/6.

This model (Figure 1C) has advantages and disadvantages. As mentioned, it addresses the issue of the location of the binding site for the phospholipid headgroup, an issue which is sidestepped by models that only propose gates. While it does not define the boundaries of the binding site and associated cavity, it proposes a general location which can be the subject of further investigation. The entrance site for phospholipids is defined by the E1261K mutation, and the exit would be near the Y618 residue.

Future prospects:

The current models, and particularly the external versus internal path models, are not readily reconciled with each other. Because the methodologies used to define them are very different, the disparity in the results suggests that one or more of these methods (and perhaps all of them) are poor instruments for addressing the problem of where the phospholipid passes through the membrane. Use of indirect assays of transport, ranging from relatively closely related (ATPase activity) to very distantly related (PS location in the cell), may account for some of the problem, and even when the enzyme activity is directly measured, there are still discrepancies of 1-2 orders of magnitude in such basic measurements as apparent Km. While these questions will finally be settled by structural determinations, that day is not obviously in prospect, and until then, additional measurements, new assays, and careful comparison of the behavior of the same mutations in different assays and different members of the family will be required to settle these issues.

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Figure Legend:

Figure 1: Location of mutations supporting trajectory models, visualized on the structure for the proton pump (3B8C). (A) Mutations supporting two gate model [28, 32, 35]; (B) Mutations supporting central Ca path model [26, 27]; (C) Mutations supporting proton pump cavity model

[29]

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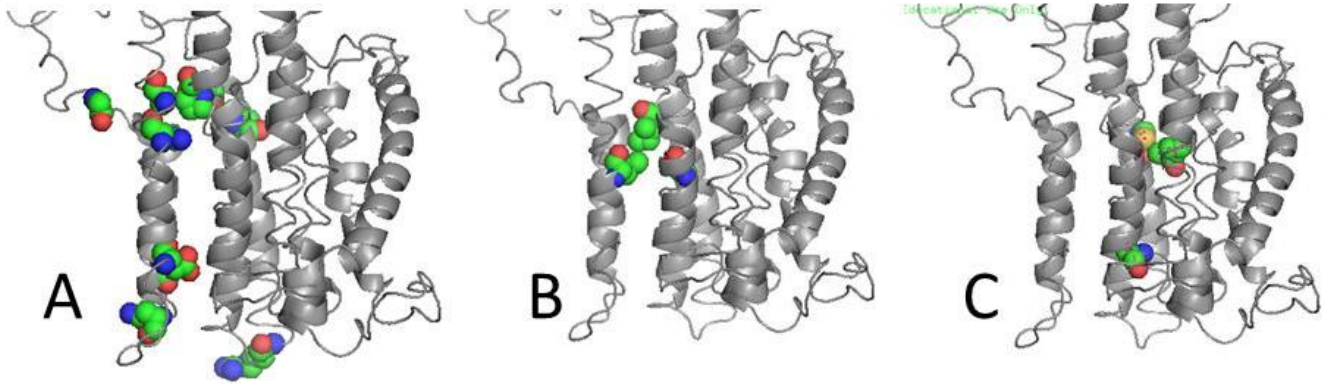
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