Specific phospholipid binding to Na,K-ATPase at two distinct sites

Michael Habeck*, Einat Kapri-Pardes*, Michal Sharon*, and Steven J. D. Karlsh*a,

*Department of Biomolecular Sciences, Weizmann Institute of Science, Rehovot 7610001, Israel

Edited by H. Ronald Kaback, Departments of Physiology and Microbiology, Immunology, and Molecular Genetics and the Molecular Biology Institute, University of California, Los Angeles, CA and approved February 7, 2017 (received for review December 18, 2016)

Membrane protein function can be affected by the physical state of the lipid bilayer and specific lipid–protein interactions. For Na,K-ATPase, bilayer properties can modulate pump activity, and, as observed in crystal structures, several lipids are bound within the transmembrane domain. Furthermore, Na,K-ATPase activity depends on phosphatidylserine (PS) and cholesterol, which stabilize the protein, and polyunsaturated phosphatidylcholine (PC) or phosphatidylethanolamine (PE), known to stimulate Na,K-ATPase activity. Based on lipid structural specificity and kinetic mechanisms, specific interactions of both PS and PC/PE have been inferred. Nevertheless, specific binding sites have not been identified definitively. We address this question with native mass spectrometry (MS) and site-directed mutagenesis. Native MS shows directly that one molecule each of 18:0/18:1 PS and 18:0/20:4 PC can bind specifically to purified human Na,K-ATPase (α7β2). By replacing lysine residues at proposed phospholipid-binding sites with glutamines, the two sites have been identified. Mutations in the cytoplasmic αL8–9 loop destabilize the protein but do not affect Na,K-ATPase activity, whereas mutations in transmembrane helices (TM), αTM2 and αTM4, abolish the stimulation of activity by 18:0/20:4 PC but do not affect stability. When these data are linked to crystal structures, the underlying mechanism of PS and PC/PE effects emerges. PS (and cholesterol) bind between αTM 8, 9, 10, near the FXDY subunit, and maintain topological integrity of the labile C terminus of the α subunit (site A). PC/PE binds between αTM2, 4, 6, and 9 and accelerates the rate-limiting E-P-E P conformational transition (site B). We discuss the potential physiological implications.

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he biological membrane is a dynamic unit of proteins and lipids. The lipid part is composed of steroids, sphingolipids, and glycerophospholipids; variations in head group structure, acyl chain length, and degree of unsaturation lead to the formation of hundreds of types of lipids (1). This complexity is not required for bilayer formation but provides second messengers, allows recognition processes, and maintains the activity of membrane proteins, which may depend on specific lipids (2). The lipid composition of membranes determines the hydrophobic thickness, fluidity, curvature, and lateral pressure profiles (3). These bulk properties may affect membrane protein function in a nonstoichiometric manner and are referred to as “nonspecific protein–lipid interactions.” Lipids associated weakly with protein transmembrane segments are termed “annular lipids” (4), and although they have restricted motion, they can exchange quickly with bulk lipids. Site-specific lipid binding is characterized by longer residence times at sites formed between transmembrane helices or protein subunits. These lipids are stabilized by polar interactions between head groups and charged amino acids at the water–lipid interface and hydrophobic interactions between acyl chains and aromatic or hydrophobic side chains of amino acids in transmembrane segments (2). Lipids observed in membrane protein structures are often copurified from the membrane, having withstood detergent solubilization, thus indicating tight binding. Because the functional roles of these lipids, such as modulation of kinetic properties, usually cannot be inferred, they are often considered relevant only for the structural integrity of membrane proteins (5).

Na,K-ATPase is a primary active transporter of the P-type ATPase family that maintains the electrochemical gradients for Na and K in animal cells by exchanging three intracellular Na ions for two extracellular K ions (6). It has long been known that Na,K-ATPase activity depends on phosphatidylserine (PS) and cholesterol (7, 8), and recent biochemical studies suggest that both lipids interact specifically with Na,K-ATPase (9, 10). Furthermore, seven phospholipids and three cholesterol molecules have been identified in crystal structures at three different sites (referred to hereafter as sites “A,” “B,” and “C”) (Fig. 1, Inset) (11, 12), but their functional importance has remained elusive. Analysis of the effects of phospholipids and cholesterol on recombinant Na,K-ATPase purified in mixed detergent–lipid-protein micelles in the absence of a lipid bilayer allows the detection of specific lipid–protein interactions. For example, PS (optimally 18:0/18:1 PS) and cholesterol stabilize the Na,K-ATPase against thermal- and detergent-mediated inactivation (9). By contrast, polyunsaturated phosphatidylcholine (PC) and phosphatidylethanolamine (PE) (optimally 18:0/20:4 or 18:0/22:6 PC/PE) stimulate Na,K-ATPase activity but do not stabilize the protein (13). The structural specificity of the phospholipids is indicative of specific Na,K-ATPase–PS and Na,K-ATPase–PC/PE interactions. Mutation of three residues in transmembrane (TM) helices αTM8, 9, and 10 of the thermolabile α7 isosform to the corresponding α7 residues (V918, F955, and P979) (Fig. 1) significantly stabilized α7 by increasing the apparent affinity for 18:0/18:1 PS (1-stearyl-2-oleoyl-sn-glycero-3-phospho-L-serine, SOPS) and FXYD1 and increasing the stabilizing effect of cholesterol (10, 14). This finding indicated that both 18:0/18:1 PS and cholesterol are bound between helices αTM8–10 and the FXYD subunit, all mutually interacting to stabilize the protein. The 18:0/18:1 PS in site A does not affect Na,K-ATPase activity per se. In crystal structures two cholesterol and four phospholipid molecules have been identified near the C terminus of the α-subunit in site A

Significance

Activity and structural integrity of membrane proteins can be regulated by physical properties of the bilayer or specific lipid–protein interactions. This work shows that key properties of the Na,K-ATPase are modulated independently by specific binding of 18:0/18:1 phosphatidylserine (PS) and 18:0/20:4 phosphatidylcholine (PC) in the absence of a bilayer. PS stabilizes the protein, and PC/PE phosphatidylethanolamine (PE) stimulates Na,K-ATPase activity. We characterized effects of both types of phospholipids by kinetic approaches, mutant analyses, and native MS. Modulation of Na,K-ATPase function by PS and PC/PE is defined by the phospholipid structural specificity, binding stoichiometry within two specific binding sites, and the kinetic mechanism. We provide detailed mechanistic insights, potentially with important implications for physiological regulation of active Na and K transport.

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1To whom correspondence should be addressed. Email: Steven.Karlish@weizmann.ac.il.

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Na,K-ATPase lipid-binding sites: the location of functionally relevant β inhibition of Na,K-ATPase does not ± with the theoretical mass of the αβ18:0/18:1 PS complex (151,272 Da). We added 10, 30, and 50 μg/mL of 18:0/18:1 PS. The 50 μg/mL addition sufficed to produce a maximal effect. The Ka for 18:0/18:1 PS is in the range 20–30 μg/mL (25–38 μM), in line with that required to stabilize the enzyme in biochemical experiments (9, 10, 18). Overnight incubation with 18:0/18:1PS did not affect activity. MS did not detect cholesterol, presumably because it dissociated from the αβ complex on the size-exclusion column. Dissociation of DDM micelles required relatively high activation/collision energy, which might lead to the dissociation of additional specifically associated lipids, thus precluding their detection. Thus, we replaced DDM with the shorter UDM, which has a threefold higher critical micelle concentration and allows the acquisition of spectra at lower activation energy (Fig. 3d). The concentration of UDM (0.6 mg/mL) plus SOPS (30 μg/mL) was optimized to give maximal resolution of MS spectra and to avoid an excess of UDM, which inactivates the Na,K-ATPase and precludes the observation of well-defined spectra. Under these conditions six molecules of PS were observed (Fig. 3b; masses are given in SI Appendix, Table S2). However, all but one of these lipids was easily dissociated upon increase of collision energy, strengthening the notion of specific binding of only one molecule of PS (Fig. 3c). The calculated mass of bound 18:0/18:1 PS was 787 ± 5 Da (n = 7), compared with the theoretical mass of 789 Da. Other SOPS molecules observed initially may represent annular or weakly associated lipids.

To define the location of the specifically bound PS molecule, we mutated lysine residues to neutral glutamines, singly or together, at the relevant sites have not been clearly identified. The current study addresses this gap in our knowledge by combining kinetic approaches with mutant analyses and native MS and by linking structural and biochemical data.

**Results**

Detection of specific effects of lipids on Na,K-ATPase from *Pichia pastoris* is based on the replacement of endogenous yeast lipids by defined lipids solubilized in non-denaturing detergents. Solubilization is achieved by extensive washing of the His-tagged Na,K-ATPase bound to Co beads with buffers supplemented with lipids and detergents before elution of the enzyme. For this study Na,K-ATPase αβ1 was purified in a mixture of 18:0/18:1 PS, cholesterol, and octaethylene glycol monododecyl ether (C12E8), undecyl-maltopyranoside (UDM), or dodecyl-maltopyranoside (DDM). The specific Na,K-ATPase activity was not significantly different when using C12E8 or maltosides.

PS and cholesterol are essential for preserving Na,K-ATPase activity, but PS has not been resolved in crystal structures. To obtain direct proof of specific binding of PS and, in particular, to address the question of lipid-binding stoichiometry, we have applied native MS. Native MS analysis of membrane proteins is based on the transfer of protein–detergent micelles in the gas phase and the removal of the detergent molecules by collisional activation while specific phospholipid interactions are maintained (15, 16). Spectra were obtained following the purification of the protein in DDM or UDM and a size-exclusion chromatography step, which isolated the Na,K-ATPase monomer peak from aggregated proteins, a small fraction of higher oligomers, and excess lipid-detergent micelles (SI Appendix, Fig. S1f). The running buffer was supplemented with 10 μg/mL 18:0/18:1 PS in 0.25 mg/mL DDM, which preserved 70% of Na,K-ATPase activity. The mass spectra indicated a major charge series with an apparent mass of 150,475 ± 30 Da and a minor charge series with apparent mass of 159,312 ± 88 Da (Fig. 2c). These masses are in good agreement with the theoretical mass of the αβ18:0/18:1 PS complex (151,232 Da) complexes. Alkyl maltosides tend to dissociate the FXYD proteins from the αβ complex (17). C12E8 does not have this effect (18) but is incompatible with MS, probably because of sample inhomogeneity caused by the presence of higher oligomers in addition to αβ monomers (19). Thus, the present work on lipid binding is limited to the UDM- or DDM-soluble FXYD-free αβ complexes. An adduct peak was observed at the major charge series with a 795-Da mass shift, suggesting binding of a PS molecule. After 18:0/18:1 PS was added to the sample and allowed to equilibrate overnight, a new peak emerged with an apparent mass of 151,272 ± 23 Da (Fig. 2d), in agreement with the expected mass of an αβ18:0/18:1 PS complex (151,232 Da). We added 10, 30, and 50 μg/mL of 18:0/18:1 PS. The 50 μg/mL addition sufficed to produce a maximal effect. The Ka for 18:0/18:1 PS is in the range 20–30 μg/mL (25–38 μM), in line with that required to stabilize the enzyme in biochemical experiments (9, 10, 18). Overnight incubation with 18:0/18:1PS did not affect activity. MS did not detect cholesterol, presumably because it dissociated from the αβ complex on the size-exclusion column. Dissociation of DDM micelles required relatively high activation/collision energy, which might lead to the dissociation of additional specifically associated lipids, thus precluding their detection. Thus, we replaced DDM with the shorter UDM, which has a threefold higher critical micelle concentration and allows the acquisition of spectra at lower activation energy (Fig. 3d). The concentration of UDM (0.6 mg/mL) plus SOPS (30 μg/mL) was optimized to give maximal resolution of MS spectra and to avoid an excess of UDM, which inactivates the Na,K-ATPase and precludes the observation of well-defined spectra. Under these conditions six molecules of PS were observed (Fig. 3b; masses are given in SI Appendix, Table S2). However, all but one of these lipids was easily dissociated upon increase of collision energy, strengthening the notion of specific binding of only one molecule of PS (Fig. 3c). The calculated mass of bound 18:0/18:1 PS was 787 ± 5 Da (n = 7), compared with the theoretical mass of 789 Da. Other SOPS molecules observed initially may represent annular or weakly associated lipids.

To define the location of the specifically bound PS molecule, we mutated lysine residues to neutral glutamines, singly or together, at the

![Fig. 1. Na,K-ATPase lipid-binding sites: the location of functionally relevant phospholipids and cholesterol in sites A and B, as detected in this study. Lysine residues at the membrane–water interface mutated in this study are shown in stick representation. V920, F956, and P981, previously shown to stabilize Na,K-ATPase, are indicated also.](image-url)

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**References**


cytoplasmic side of membrane crevices expected to harbor functionally relevant phospholipids [Lys945 and Lys943 in the αL–S–9 loop (site A), Lys146 in αTM2, and Lys342 in αTM4 (site B); Fig. 1]. Single and double mutants (αK943Q, αK945Q, αK943Q/K945Q, αK146Q, αK342Q, and αK146Q/K342Q) were introduced by site-directed mutagenesis and transformed into P. pastoris as αMutHis6[α]. As indicated in SI Appendix, Table S1, which documents the ouabain-binding capacity of the membranes, three single mutants—αK943Q, αK945Q, and αK342Q—were expressed only weakly and could not be purified. By contrast, expression levels of mutants αK943Q/K945Q, αK146Q, and αK146Q/K342Q reached ∼50% of WT levels. These three mutants and WT Na,K-ATPase were purified in a mixture of C60Eo, 18:0/18:1 PS and cholesterol and were used either for stability assays (Fig. 4) or with added 18:0/20:4 PC to determine the stimulatory effect on Na,K-ATPase activity and rates of conformational transitions (Fig. 5). Specific ATPase activities of batch-purified enzyme were 15.3 ± 0.7 μmol P·mg⁻¹·min⁻¹ for WT α1 and 10.7 ± 0.8, 11.8 ± 0.7, and 10.9 ± 0.8 μmol P·mg⁻¹·min⁻¹ for αK943Q/K945Q, αK146Q, and αK146Q/K342Q.

Fig. 4 shows the effect of mutations on the inactivation of Na,K-ATPase activity by excess C60Eo, which is thought to displace 18:0/18:1 PS (see figure 1 in ref. 18) but is unable to mimic the stabilizing function, thus leading to inactivation. By comparison with the WT, the mutant K943Q/K945Q showed a large increase in sensitivity to C60Eo with apparent K0.5 of inactivation of 0.09 mM C60Eo compared with 0.65 mM for the WT. By contrast, both αK146Q and αK146Q/K342Q mutants show sensitivity similar to that of the WT with a K0.5 of about 0.75 mM C60Eo, thus serving as negative controls for the selective effect of the K943Q/K945Q mutant on stability.

Cholesterol and SOPS are the only lipids essential for maintaining Na,K-ATPase function, but they are not sufficient to sustain maximal activity. As shown recently, PC or PE with saturated fatty acyl chains in sn-1 and polyunsaturated fatty acyl chains in sn-2 stimulate Na,K-ATPase activity by 50–60% (14). Fig. 5 compares the Na,K-ATPase activity of the WT and mutant proteins purified in the presence of 18:0/18:1 PS and cholesterol without or with 18:0/20:4 PC or 18:0/18:1 PC. In agreement with previous findings, 18:0/20:4 PC stimulated the activity of WT α1β1 by 44 ± 3% compared with the control, whereas 18:0/18:1 PC did not stimulate activity. We investigated the effect of 18:0/20:4 PC on the Na,K-ATPase activity of the lysine mutants. As shown in Fig. 5B, stimulation of αK943Q/K945Q by 18:0/20:4 PC was comparable to stimulation of the WT (37 ± 7%). By contrast, the double mutation K146Q/K342Q completely abolished stimulation by 18:0/20:4 PC, and K146Q only showed about 10% stimulation by 18:0/20:4 PC. Na,K-ATPase transports ions by cycling between two principle conformations, E1 and E2 (SI Appendix, Fig. S2), and the rate-limiting steps are the conformational transitions E2P→E1P and E2(2K)→E1. Rates of conformational transitions can be measured by stopped-flow fluorescence using the electrochromic shift dye RH421 (14). The E2P→E1P transition is triggered by mixing Na,K-ATPase in the presence of Na with ATP (E2NαATP→E2P+3NαADP). We have shown previously that the stimulatory lipid brain PE accelerates the rate of the E2P→E1P transition (14). For stopped-flow experiments the mutant αK146Q/K342Q was purified in the absence (control) and presence of 18:0/20:4 PC (Fig. 5C). In agreement with the lack of stimulatory effect of 18:0/20:4 PC on Na,K-ATPase activity, the traces are virtually overlapping, with rates determined as 99 ± 9% for the control and 87 ± 7% for the mutant purified with 18:0/20:4 PC.

In short, Figs. 4 and 5 show that mutations of lysine residues in site A (αK943Q/K945Q) increase sensitivity to destabilization by C60Eo but do not affect the stimulation of activity by 18:0/20:4 PC, whereas mutations in site B (αK146Q, αK146Q/K342Q) prevent the stimulation of activity by 18:0/20:4 PC but do not affect stability. These findings confirm that the effects are separate and selective, resulting from specific lipid binding in the distinct sites A and B (Fig. 1).

Next, we used native MS to demonstrate directly the specific binding of 18:0/20:4 PC to the Na,K-ATPase. [We used 18:0/20:4 PC in...
K943Q/K945Q substitutions increase sensitivity to detergent inactivation. WT Na⁺,K⁺-ATPase and its mutational variants α₁K146Q, α₁K146Q/K342Q, and α₁K342Q/K945Q were incubated in the presence of the indicated concentrations of C₁₂E₆ for 10 min at 37 °C before residual activity was measured. Error bars represent the SEM. n = 3.

Discussion

When a membrane protein is purified in its native membrane or reconstituted into a model bilayer, as is often necessary in order to maintain activity, it is difficult to distinguish whether functional effects of different lipids result from specific protein–lipid interactions or from physical forces associated with nonspecific bilayer interactions. Thus, the different types of protein–lipid interaction have been a matter of extensive debate for channels (20, 21), transporters (22), G-protein–coupled receptors (23), and P-type ATPases (SI Appendix, Supplementary Discussion). By contrast, when the membrane protein is purified in soluble mixed protein–lipid–detergent micelles in the absence of a lipid bilayer, the analysis is greatly simplified. Indeed, the ability to measure the Na⁺,K⁺-ATPase activity of the soluble Na⁺,K⁺-ATPase–lipid micelles allowed us to show that stability and activity are independently modulated by three different classes of lipids (10, 14). However, to understand the mechanism by which specifically bound lipids modulate Na⁺,K⁺-ATPase, it is essential to identify the sites and determine the stoichiometry of lipid binding. The present study provides biochemical and structural insights and unequivocally defines two lipid-mediated effects. The effects were inferred previously by their structural specificity for different lipids and the kinetic mechanisms (9, 14). Although these data were convincing, they were still indirect. By combining native MS and biochemical analysis of mutants, we could demonstrate directly both the stoichiometry of lipid binding and the location of sites A and B (Fig. 1).

As shown in previous work, optimal stabilization of the enzyme depends on specific binding of 18:0/18:1 PS, cholesterol, and the FXYD subunit (10, 14). These observations provided clear evidence for a specific SOPS-binding pocket between α TM9, 10 and TMFXYD in the C-terminal domain of the α-subunit (site A). They imply that SOPS, cholesterol, and FXYD all interact with each other to stabilize the protein. The molecular structure of Na⁺,K⁺-ATPase with bound lipids in site A (Fig. 1, Inset) suggested that a particular phospholipid facing the cytoplasmic surface could be the relevant one. Accordingly, we mutated Lys943 and Lys945 in the cytoplasmic-facing L8–9 loop to neutral glutamines. The two lysine residues are conserved among Na⁺,K⁺-ATPase isoforms (except that Lys943 is an arginine in α₄) and are the only two charged residues at the likely site of interaction with the phosphate and carboxyl groups of the SOPS. As predicted, these mutations significantly destabilized Na⁺,K⁺-ATPase against detergent inactivation, whereas the other mutations in TM2 and TM4 had no effect on stability. We conclude that SOPS is indeed bound to Lys943 and Lys945 in L8–9. To determine the number of specifically bound SOPS molecules, we performed native MS experiments. The optimal conditions required for structural MS experiments were somewhat suboptimal for the preservation of Na⁺,K⁺-ATPase activity; namely, size-exclusion chromatography in the presence of high detergent–lipid ratios leads to the dissociation of FXYD1 and cholesterol and to some loss of activity (~30%). Nevertheless, we observed specific binding of one molecule of 18:0/18:1 PS. Thus, we conclude that the one 18:0/18:1 PS molecule binds to the C-terminal domain of the Na⁺,K⁺-ATPase α-subunit near the FXYD subunit and a cholesterol (see site A in Fig. 1). [In the Na⁺,K⁺-ATPase structure, Protein Data Bank (PDB) ID code: 3WGV (11), three cholesterol molecules were identified, including two located between α TM6–10 (Chl3 and Chl2 in Fig. 1). Chl3 is inferred to stabilize the protein via interactions with preference to 18:0/20:4 PE because of its higher mass (810 Da) versus 768 Da, respectively), allowing a readier distinction from bound 18:0/18:1 PS (789 Da). The enzyme was purified by size-exclusion chromatography in the presence of 0.6 mg/mL UDM and 30 μg/mL 18:0/18:1 PS before increasing amounts of 18:0/20:4 PC were added. Samples then were allowed to equilibrate for 8 h on ice. As shown in ref. 13, overnight incubation with the unsaturated phospholipid increased Na⁺,K⁺-ATPase activity. Spectra were acquired with activation conditions tuned to dissociate most of the annular bound PS allowing but not binding of PC (Fig. 6A). The addition of 18:0/20:4 PC led to the concentration-dependent appearance of a peak with a mass corresponding to the mass of two phospholipids bound to the apo-αβ₁ complex (152,078 ± 24 Da; the theoretical mass αβ₁ 18:0/18:1 PS 152,042 Da). The calculated mass of the bound 18:0/20:4 PC was 808 ± 7 Da (n = 4) compared with the theoretical mass of 810 Da. The increase in relative peak intensity was fitted to a Hill function with apparent E₅₀ of 11.9 μM and a Hill coefficient of 1.05, indicating, again, that only one molecule of 18:0/20:4 PC binds specifically to the Na⁺,K⁺-ATPase (Fig. 6B). As a control for specificity of its binding, we replaced 18:0/20:4 PC with 18:0/18:1 PC, which does not stimulate pump activity (Fig. 5A). Indeed, no significant binding of 18:0/18:1 PC was observed upon the addition of 30 μg/mL 18:0/20:4 PC (Fig. 6C), as seen by comparing the relative peak heights with those of the control spectrum without added 18:0/20:4 PC (Fig. 6A).

Attempts to look at mutants by MS were unsuccessful. An unexpected effect explains this observation (SI Appendix, Fig. S1B). Size-exclusion chromatography shows that all the mutants tended to aggregate or form higher oligomers. The same pattern was observed when WT Na⁺,K⁺-ATPase was purified in detergent alone without PS and cholesterol. Thus, these observations also indicate that the bound phospholipids maintain the functional monomeric state of the Na⁺,K⁺-ATPase.

Fig. 4. K943Q/K945Q substitutions increase sensitivity to detergent inactivation. WT Na⁺,K⁺-ATPase and its mutational variants α₁K146Q, α₁K146Q/K342Q, and α₁K342Q/K945Q were incubated in the presence of the indicated concentrations of C₁₂E₆ for 10 min at 37 °C before residual activity was measured. Error bars represent the SEM. n = 3.

Fig. 5. Lysine substitutions in M2 and M4 prevent stimulation by 18:0/20:4 PC. (A) 18:0/20:4 PC but not 18:0/18:1 PC stimulates pump activity. Activity was normalized to the reference sample purified in the presence of 18:0/18:1 PS and cholesterol (SOPS). (B) Mutation of lysines in M2 and M4 (K146Q and K146Q/K342Q) but not in the L8–9 loop (K943Q/K945Q) abolish stimulation by 18:0/20:4 PC. (C) Stopped-flow traces of the E₃₃P₃ Na⁺,ATP → E₃₃P₃ Na⁺,ADP conformational transition for α₁K146Q/K342Q purified with and without 18:0/20:4 PC. Error bars represent the SEM. n ≥ 3. *P < 0.05, ***P < 0.0005.
bulk bilayer properties in intact membranes. Polyunsaturated lipids have been shown to reduce Na,K-ATPase turnover in liposomes enriched in 22:6 PC (27). Conversely, Na,K-ATPase turnover rates in avian and mammalian cardiac membranes were positively correlated with the fraction of docosahexaenoic acid (22:6) in phospholipids (28), an effect attributed to an increase in the area of the phospholipids as the polyunsaturated acyl chain composition increased (29). However, given the inhibitory effect of 22:6 PC in reconstituted liposomes, it is more likely that the increased Na,K-ATPase activity was caused by the specific stimulatory interactions of the 22:6-containing phospholipid rather than by an effect of molecular packing. The activity of Na,K-ATPase purified in a mixture of 18:0/18:1 PS, cholesterol, and 18:0/20:4 or 18:0/22:6 PC/PE is comparable to the activity of purified renal Na,K-ATPase (30). Nevertheless, the physiological importance of specific protein–lipid interactions is not well established, in part because of the difficulty in determining changes in the lipid composition within cells. In one interesting case imaging MS has shown that arachidonic acid–PC and docosahexaenoic acid–PC are enriched across a proximal-to-distal gradient of cultured neurons (31). Na,K-ATPase activity is vital for neuronal function, particularly at nerve endings where it maintains the Na gradients required for neurotransmitter reuptake. It is intriguing to speculate that the gradient of fatty acyl chain unsaturation may have increased Na,K-ATPase activity along the axon by the specific interaction described here.

An unexpected finding in this study was that the lysine substitutions caused aggregation of Na,K-ATPase. The mutations did not significantly affect Na,K-ATPase activity and allowed biochemical studies, but the mutated proteins did not allow analysis by MS. These findings indicate that the specific binding of lipids protects against nonspecific interactions of transmembrane helices and protein aggregation.

In conclusion, by combining information from mutational analysis, biochemical data, and native MS data, it was determined that Na,K-ATPase with available crystal structures, a rather detailed picture of specific lipid-binding sites of the Na,K-ATPase has emerged (Fig. 1). Two independent binding sites have been definitively identified. Site A is formed by αTM8–10 and the FXYD transmembrane helix and harbors one molecule of 18:0/18:1 PS and one cholesterol molecule, which together stabilize but do not per se affect Na,K-ATPase activity. At a second site, B, located between αM2, 4, 6, and 9, 18:0/20:4 or 18:0/22:6 PC/PE bind and stimulate activity by accelerating the E1–E2–E3 conformational transition but do not affect stability. The different effects of lipid classes at the separate sites are independent and modulate distinct properties of Na,K-ATPase. A further point of interest is that the phenomenon of selective lipid binding may not be restricted to Na,K-ATPase. Crystal structures of the closely related sarcoplasmic reticulum Ca-ATPase reveal bound lipids between the same transmembrane segments (analogous to sites A and B) (32). These lipids may have effects on the stability and activity of Ca-ATPase similar to those proposed for Na,K-ATPase (see SI Appendix, Supplementary Discussion for a discussion of lipid binding in Ca-ATPase and other P-type ATPases).

Since the description of the fluid-mosaic model by Singer and Nicolson (33), the effects of the physical state of the bilayer on membrane proteins have been studied in detail (3), but the understanding of how specifically bound lipids modulate enzymatic properties is still lacking. Identifying lipid-binding sites and their mechanism of modulation of Na,K-ATPase shows that specific interactions play a key role compared with bulk bilayer interactions. This work may provide a framework for the study of specific protein–lipid interactions for other P-type ATPases, such as P4-ATPases that flip PS and PE across the membrane to maintain bilayer asymmetry (34).

**Materials and Methods**

**Reagents.** Phospholipids were purchased from Avanti Polar Lipids, cholesterol was purchased from Sigma Aldrich, and all other reagents were used at the highest available purity.

**Mutagenesis.** Mutations were introduced into the PhlD2 vector harboring human α1 and His10-β, by overlap-extension PCR (SI Appendix, SI Materials and Methods) (35). *P. pastoris* SMD1165 was transformed, and clones were selected as described (36).
Enzyme Purification. Na,K-ATPase and FXYD1 were purified by metal affinity chromatography using a batch protocol or gravity columns in a mixture of 18/0/70/3 PS, cholesterol, and C18, EDP, DDM, or UDM as detergent (see SI Appendix, SI Materials and Methods for details) (14).

Biochemical Assays. ATPase activity was measured in the presence of 120 mM NaCl, 20 mM KCl, and 1 mM ATP at 37 °C using the malachite green assay for phosphate detection (PicolorLock; Innovia Bioscience). Rates of the E2,Na3,E3,P transition were measured using an Applied Photophysics SX20 stopped-flow device by mixing 10 μg/mL Na,K-ATPase noncovalently labeled with RH421 with 2 mM ATP in the presence of 120 mM NaCl (14).

Native MS. For native MS experiments eluted Na,K-ATPase was dialyzed and deglycosylated by EndoH, and aggregated proteins were removed by ultracentrifugation. The sample was then concentrated to 5–8 mg/mL using Vivaspun 100-kDa centrifugal filters. The buffer was exchanged with 400 mM ammonium acetate (pH 7.8) or 200 mM ammonium acetate, 4 mM ethylenediamine supplemented with 0.25 mg/mL DDM or 0.6 mg/mL UDM plus 10–30 mM 18:0/18:1 PS by size exclusion chromatography (Superdex 200 10/300 column). Mass spectra were acquired on a Synapt high-definition MS (HDMS) instrument (Waters) under native-like conditions. For lipid-binding experiments concentrated stock solutions of 18:0/18:1 PS or 18:0/20:4 PC were obtained by fitting the peaks to a series of Gaussian functions.

Data Analysis. Stopped-flow fluorescence traces were fitted with a double exponential function: $F = A_1 e^{-t/\tau_1} + A_2 e^{-t/\tau_2} + c$, where $A$ is the amplitude, $\tau$ is the rate, and $c$ the equilibrium fluorescence level after completion. Peak intensities of apo-Na,K-ATPase and lipid-bound Na,K-ATPase were obtained by fitting the peaks to a series of Gaussian functions.

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