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Roles of transmembrane segment M1 of Na^+,K^+-ATPase and Ca^{2+}-ATPase, the gatekeeper and the pivot

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Abstract In this review we summarize mutagenesis work on the structure–function relationship of transmembrane segment M1 in the Na^+,K^+-ATPase and the sarco(endo)plasmic reticulum Ca^{2+}-ATPase. The original hypothesis that charged residues in the N-terminal part of M1 interact with the transported cations can be rejected. On the other hand hydrophobic residues in the middle part of M1 turned out to play crucial roles in Ca^{2+} interaction/occlusion in Ca^{2+}-ATPase and K^+ interaction/occlusion in Na^+,K^+-ATPase. Leu^{65} of the Ca^{2+}-ATPase and Leu^{99} of the Na^+,K^+-ATPase, located at homologous positions in M1, function as gate-locking residues that restrict the mobility of the side chain of the cation binding/gating residue of transmembrane segment M4, Glu^{309}/Glu^{329}. A pivot formed between a pair of a glycine and a bulky residue in M1 and M3 seems critical to the opening of the extracytoplasmic gate in both the Ca^{2+}-ATPase and the Na^+,K^+-ATPase.

Keywords Na^+,K^+-ATPase · Ca^{2+}-ATPase · Transmembrane segment M1 · Ion occlusion · Leucine · P-type ATPase · Gating

All numbering of Na^+,K^+-ATPase amino acid residues in this article refers to the sequence of the rat α_1-isoform.

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Introduction

The Na^+,K^+-ATPase and the sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) are prominent members of the family of cation transporting P-type ATPases, which in addition include H^+,K^+-ATPase, heavy metal transporting ATPases, plasma membrane and secretory pathway Ca^{2+}-ATPases, as well as plant and yeast H^+-ATPases and phospholipid flippases. The distinguishing biochemical feature of P-type ATPases is the formation during their reaction cycle of a covalent acylphosphate enzyme intermediate, resulting from the transfer of the γ-phosphoryl group of ATP to a conserved aspartyl residue within the protein (Pedersen and Carafoli 1987). The structural similarities among P-type ATPases reside within one main chain (denoted α-subunit in Na^+,K^+- and H^+,K^+-ATPases), although in some of the P-type ATPases, the function and targeting to specific cellular locations depend on interaction of the main chain with accessory subunits, the β-subunit in case of the Na^+,K^+- and H^+,K^+-ATPases. All P-type ATPases contain conserved cytoplasmic sequences associated with the catalytic site, including the DKTG-motif with the phosphorylated aspartic acid residue and the TGES motif containing a glutamate required for dephosphorylation (Clausen et al. 2004). The Na^+,K^+-ATPase is most closely related to the H^+,K^+-ATPase (60% overall amino acid sequence identity) and SERCA (~30% identity and 65% similarity). In fact, the homology between SERCA and the Na^+,K^+-ATPase is more pronounced than between SERCA and the plasma membrane Ca^{2+}-ATPase (Sweadner and Donnet 2001).

The P-type ATPases seem to operate by a common mechanism involving two major conformations E_1 and E_2, both of which exist in a phosphorylated and a dephosphorylated state. The reaction sequence $E_1 + 3Na^+_{cyt} \rightarrow Na_3E_1 \rightarrow [Na_3]E_1P \rightarrow E_2P + 3Na^+_{ext} \rightarrow E_2P + 2K^+_{ext} \rightarrow K_2E_2P \rightarrow$

$[K_2]E_2 \rightarrow E_1 + 2K^+_{cyt}$ describes how the transitions between the Na^+,K^+-ATPase conformational states lead to ATP hydrolysis coupled with sequential ion translocation across the membrane (Post et al. 1972; Glynn 1993). In E_1 and E_2P, the enzyme binds Na^+ and K^+ from the cytoplasmic and extracellular sides of the membrane, respectively. In $[Na_3]E_1$P and $[K_2]E_2$, the ions are in transit, bound in so-called "occluded" states (indicated by brackets) where they have no access to the medium on either side of the membrane due to closure of the cytoplasmic gate as well as the extracellular gate (Vilsen et al. 1987; Glynn 1993). In the analogous reaction cycle of SERCA, two Ca^{2+} ions are translocated towards the lumen of the sarco(endo)plasmic reticulum in exchange for two to three protons for each ATP being hydrolyzed, with Ca^{2+} and H^+ being occluded in E_1P and E_2, respectively.

The Na^+,K^+-ATPase α-subunit and the Ca^{2+}-ATPase are both made up of 10 transmembrane helices, M1–M10, and a cytoplasmic part consisting of three domains denoted A- (actuator), N- (nucleotide-binding), and P- (phosphorylation) domain (Toyoshima et al. 2000; Hu and Kaplan 2000; Sweadner and Donnet 2001). Residues in the transmembrane segments M4, M5, M6, M8, and possibly M9 (Na^+,K^+-ATPase) donate oxygen ligands for binding of the transported cations (Clarke et al. 1989a; Vilsen and Andersen 1992b, 1998; Jewell-Motz and Lingrel 1993; Andersen and Vilsen 1994; Arguello and Kaplan 1994; Vilsen 1995a; Kuntzweiler et al. 1996; Blostein et al. 1997; Pedersen et al. 1998; Toyoshima et al. 2000; Ogawa and Toyoshima 2002). Glu^{329} of M4 (Ca^{2+}-ATPase Glu^{309}) has been pinpointed as a crucial residue that gates the entrance to the cation binding pocket, both at the cytoplasmic side in the E_1 form and at the extracytoplasmic side in the E_2P form, thus presumably moving in connection with the ion translocating conformational change (Vilsen and Andersen 1998). Structural models based on X-ray crystallography of the various Ca^{2+}-ATPase intermediates have indicated that the transport associated conformational changes encompass changes in the relation of the cytoplasmic domains as well as the transmembrane segments, including a shift of M1–M2 helices in the direction perpendicular to the membrane and movement of the cytoplasmic bent part of M1 along the membrane surface (Toyoshima et al. 2000, 2004; Toyoshima and Nomura 2002; Toyoshima and Mizutani 2004; Sorensen et al. 2004; Olesen et al. 2004).

In this review we focus on our mutational studies elucidating the role of transmembrane segment M1 in the transport mechanism (Einholm et al. 2003, 2004, 2005, 2007). Before these studies the only evidence pointing to an important role of M1 was the finding of the mutation Leu^{65}Ser in SERCA2b in some patients with Darier disease (Ruiz-Perez et al. 1999), whereas no significant effects on the functional cycles of Ca^{2+}-ATPase or Na^+,K^+-ATPase had been revealed by *in vitro* mutagenesis studies of other residues in M1 (Clarke et al. 1989a, b, 1990; MacLennan et al. 1997; Askew and Lingrel 1994; Canessa et al. 1992; Price and Lingrel 1988; Price et al. 1989, 1990; Schultheis and Lingrel 1993). Generally, the sequence homology between the Ca^{2+}-ATPase and the Na^+,K^+-ATPase is rather low in M1 (Fig. 1), raising the question whether the structural and functional roles played by M1, if any, are similar or distinct.

All in all, we investigated 43 mutants with alteration to residues in M1 of the Ca^{2+}-ATPase or the Na^+,K^+-ATPase (indicated in Fig. 1). The effects of the mutations on the various partial reactions involved in ion translocation and ATP hydrolysis were analyzed by steady-state and transient kinetic methods.

Rejection of the hypothesized involvement of charged residues of the N-terminal part of M1 in the interaction with transported ions

The presence of four carboxylic acid residues in the N-terminal part of M1 of the Ca^{2+}-ATPase versus two positively charged (Lys^{86} and Arg^{89}) and one negatively charged (Glu^{83}) side chains in the same region of the Na^+,K^+-ATPase (see Fig. 1) led to the hypothesis that these M1 residues possibly together with one or more negatively charged residues of M2 and/or M3 contribute to a cation selectivity filter similar to that proposed for certain Na^+ and Ca^{2+} channels, where the ion selectivity depends on the presence of two negatively charged side chains and one positive side chain in the Na^+ channel and on four negatively charged side chains in the Ca^{2+} channel (Lipkind and Fozzard 2000, 2001). The first crystal structure of the Ca^{2+}-ATPase corresponding to Ca_2E_1 (Toyoshima et al. 2000) revealed the presence of a short channel passing between Glu^{55} (M1) and Glu^{109} (M2) on the membrane surface to Glu^{309}, the Ca^{2+} binding residue of M4. The four carboxylic acid residues of

Fig. 1 Sequence alignments (Sweadner and Donnet 2001) of the M1 region of Ca^{2+}-ATPase (*upper*) and Na^+,K^+-ATPase (*lower*). Conserved residues are indicated in red. The various mutations introduced into the Ca^{2+}-ATPase are shown above the replaced residues, whereas those introduced into the Na^+,K^+-ATPase are shown below

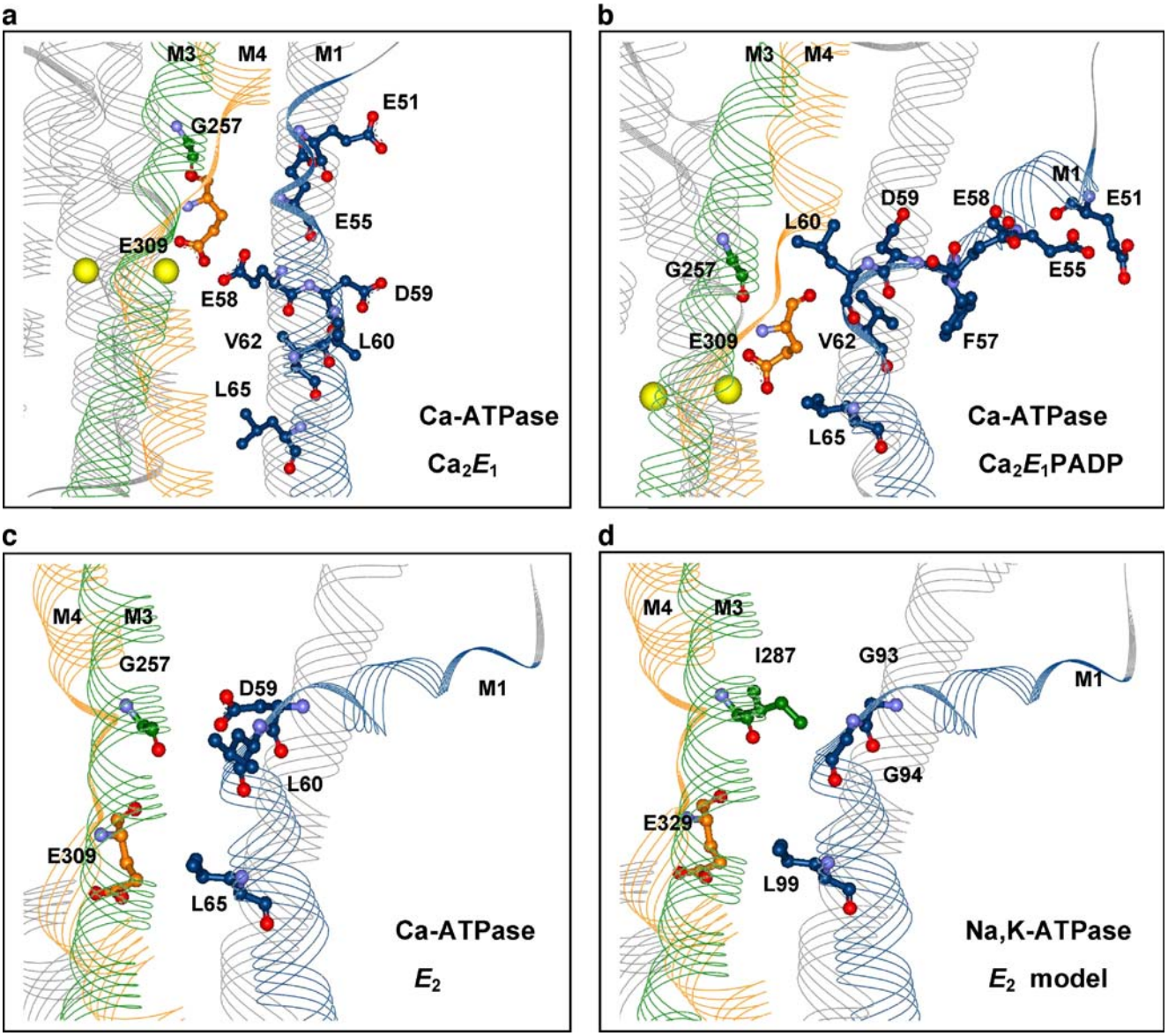


Fig. 2 Side view of transmembrane segments M1, M3, and M4 in various conformational states of the Ca^{2+}-ATPase (**a**–**c**) and the Na^+,K^+-ATPase (**d**). The cytoplasmic side is above and the luminal/extracellular side is below. **a** The first crystal structure of the Ca^{2+}-ATPase in the Ca_2E_1 form (PDB accession code 1SU4). In this structure the M1 helix is straight and Glu^{58} of M1 contacts the Ca^{2+} binding residue Glu^{309} of M4. The two bound Ca^{2+} ions are shown here and in (**b**) as yellow spheres. **b** Ca_2E_1 form with bound ADP and AlF_4^- (PDB accession code 1T5T). In this structure M1 bends and the two Ca^{2+} ions are occluded, i.e. their access to the medium is blocked. The carboxylic acid residues Glu^{51}, Glu^{55}, and Glu^{58} now occupy peripheral positions. **c** E_2 form with bound MgF_4^{2-} and thapsigargin (PDB accession code: 1WPG). **d** Na^+,K^+-ATPase model based on the Ca^{2+}-ATPase E_2 crystal structure in (**c**). The pair Leu^{60}-Gly^{257} of the Ca^{2+}-ATPase (**b** and **c**) and the pair Gly^{94}–Ile^{287} of the Na^+,K^+-ATPase (**d**) form a contact point between M1 and M3 that could function as a pivot for the movement of M1 during the ion translocation (Einholm et al. 2005). Glu^{309} (Ca^{2+}-ATPase) and Glu^{329} (Na^+,K^+-ATPase) of M4 gate the entrance to the cation binding pocket, both at the cytoplasmic side in the E_1 form and at the extracytoplasmic side in the E_2P form, thus presumably moving in connection with the ion translocating conformational change (Vilsen and Andersen 1998). The conserved leucine of M1 (Leu^{65} in Ca^{2+}-ATPase and Leu^{99} in Na^+,K^+-ATPase) functions as a gate-locking residue ("gatekeeper"), restricting the mobility of the side chain of Glu^{309}/Glu^{329}, thereby being critical to ion occlusion (Einholm et al. 2007). The figures were prepared by use of WebLab Viewer (Molecular Simulations Ltd., Cambridge, England). Carbon atoms of residues in M1, M3, and M4 are *blue*, *green*, and *orange*, respectively. Oxygen atoms are shown in *red* and nitrogen atoms in *light blue*. Residues in **a**–**c** are numbered according to the rabbit SERCA1a-isoform of the Ca^{2+}-ATPase, and residues in **d** are numbered according to the rat α1-isoform of the Na^+,K^+-ATPase

M1 (Glu^{51}, Glu^{55}, Glu^{58}, and Asp^{59}) occupy rather strategic positions along this channel (Fig. 2a), which was proposed to constitute the cytoplasmic entry pathway to the Ca^{2+} binding sites (Lee and East 2001), because the carboxylate groups might interact with the Ca^{2+} ions, guiding them to their membranous binding sites. An important role of these four acidic residues in M1 was supported by their conservation in the SERCA family of Ca^{2+}-ATPases. Furthermore, the close proximity in the Ca_2E_1 crystal structure of Glu^{58} to Glu^{309} at the Ca^{2+} binding site, with Glu^{58} likely forming a hydrogen bond to Glu^{309} (Fig. 2a) indicated that M1 might exert a direct conformational effect upon Ca^{2+} occupancy of the binding sites (Lee and East 2001).

To test the hypothesis about Glu^{51}, Glu^{55}, Glu^{58}, and Asp^{59} of the Ca^{2+}-ATPase as part of a possible Ca^{2+} access pathway and/or ion selectivity filter, we mutated these amino acids individually to the neutral leucine and to the rather bulky and oppositely charged arginine (Einholm et al. 2003, 2004). To test the corresponding hypothesis in the Na^+,K^+-ATPase, Glu^{83} was substituted with the neutral alanine and Lys^{86} and Arg^{89} with alanine and the oppositely charged glutamate (Einholm et al. 2007).

Ca^{2+}-ATPase

Neither of the replacements of Glu^{51}, Glu^{55}, and Glu^{58} affected the Ca^{2+} transport rate, the apparent Ca^{2+} affinity, or the rate of Ca^{2+} dissociation from the cytoplasmically facing Ca^{2+} sites of the Ca^{2+}-ATPase. In fact, the functional behavior of these mutants was wild type-like in all aspects, thus arguing against any direct contribution of these glutamate side chains to the initial recognition and binding of the Ca^{2+} ions (Einholm et al. 2003, 2004). This is best rationalized in terms of the Ca^{2+}-ATPase crystal structures published subsequent to the Ca_2E_1 structure. While the first Ca_2E_1 structure has a straight M1 helix (Fig. 2a), in all later crystal structures of the Ca^{2+}-ATPase, the helical structure of M1 is bended at Asp^{59}, and the region N-terminal to the bend forms a short amphipathic helix lying at the membrane interface, cf. Fig. 2 (Toyoshima et al. 2000, 2004; Toyoshima and Nomura 2002; Toyoshima and Mizutani 2004; Sorensen et al. 2004; Olesen et al. 2004; Jensen et al. 2006; Moncoq et al. 2007; Takahashi et al. 2007). Consequently, Glu^{51}, Glu^{55}, and Glu^{58}, which make up the hydrophilic surface of this amphipathic helix, all occupy peripheral positions with respect to the suggested cytoplasmic Ca^{2+} entrance, and this could explain their lack of importance in relation to Ca^{2+} interaction. The first published Ca_2E_1 crystal structure, which shows a straight M1 helix (Toyoshima et al. 2000), might actually not be a representative model of the structure of the enzyme in its native state. Because the Ca_2E_1 crystal structure does not contain bound nucleotide, the cytoplasmic domains are in an open configuration, which may affect the structure of M1 indirectly through the linker between the A-domain and M1. Fe^{2+}-catalyzed cleavage studies of the native non-crystalline state of the Ca^{2+}-ATPase have shown that the N- and P-domains approach one another in Ca_2E_1, at least temporarily (Montigny et al. 2004), and electron microscopic analysis of two-dimensional crystals of Ca_2E_1 have unveiled a less open arrangement of the cytoplasmic domains (Ogawa et al. 1998) as compared with the Ca_2E_1 structure obtained by X-ray diffraction of three-dimensional crystals (Toyoshima et al. 2000), indicating that the conformation seen in the latter crystal structure is just one out of many possible ones of the Ca_2E_1 form. This provides a compelling example of the caution needed when interpreting crystal structures that generate static pictures of selected conformations and do not provide insight into the dynamic behavior of the protein in its native environment.

In the atomic model of Ca_2E_1 with the straight M1 helix, the side chains of Glu^{58} in M1 and the Ca^{2+}-binding glutamate, Glu^{309}, in M4 are within hydrogen-bond distance (2.44 Å) (Fig. 2a). The only way such a close apposition can be permitted is by formation of a hydrogen bond between these two glutamates, implying that one of these residues is protonated. The lack of effect of the $Glu^{58}Arg$ mutation (Einholm et al. 2003, 2004) questions the existence of the Glu^{58}–Glu^{309} interaction, since the introduction of the longer and positively charged arginine at the position corresponding to Glu^{58} would be expected to result in steric collision with Glu^{309} and repulsion of the Ca^{2+} ions. The discrepancy of the functional data with the position of Glu^{58} in the Ca_2E_1 crystal structure could be explained either by a high degree of thermal mobility of the Glu^{58} side chain or by structural differences between the crystalline Ca_2E_1 form and the native non-crystalline Ca_2E_1 conformation in this region as outlined above. Whatever is the explanation, the hydrogen bond, if transiently formed, is obviously not of importance for the structural integrity of the Ca^{2+} binding sites in the native non-crystalline state. This conclusion is further substantiated by the lack of effect of the leucine substitution (Einholm et al. 2004). It should be mentioned that in two recent studies examining the ionization states of the Ca^{2+}-coordinating residues and that of Glu^{58} by continuum electrostatic calculations, it was concluded that Glu^{58} is protonated at neutral pH in the Ca_2E_1 form, but not in the E_2 form of the enzyme (Obara et al. 2005; Sugita et al. 2005). In the latter of these studies, the protonation of Glu^{58} was claimed to be of structural importance, stabilizing the Ca^{2+} sites by reducing the electrostatic repulsions between Glu^{58} and Glu^{309} in the Ca^{2+} bound form of the Ca^{2+}-ATPase (Sugita et al. 2005). In the light of the mutagenesis results (Einholm et al. 2003, 2004), it would clearly have been more relevant to perform the continuum electrostatic calculation

for an Ca_2E_1 form with a bent M1 helix, using as basis one of the Ca_2E_1 structures with bound nucleotide.

By contrast to the mutations of Glu^{51}, Glu^{55}, and Glu^{58}, arginine and leucine mutations of Asp^{59} markedly affected the Ca^{2+} binding properties of the Ca^{2+}-ATPase (Einholm et al. 2003, 2004). However, whereas mutants Asp^{59}Arg and Asp^{59}Leu both displayed a conspicuous 10-fold acceleration of the rate of Ca^{2+} dissociation from the Ca_2E_1 form toward the cytoplasmic side, Asp^{59}Ala did not affect the rate constant for Ca^{2+} dissociation. Accordingly, the apparent affinity for Ca^{2+} activation of phosphorylation was reduced in Asp^{59}Arg and wild type-like in Asp^{59}Ala. The fact that substitution of Asp^{59} with the neutral alanine left the Ca^{2+} binding properties of the Ca^{2+}-ATPase unaffected suggests that a relatively small residue is required at this position and seems to exclude a role of the negative charge on the Asp^{59} side chain in interaction with the Ca^{2+} ions. Hence, in this respect Asp^{59} is not different from Glu^{51}, Glu^{55}, and Glu^{58}.

Na^+,K^+-ATPase

In analogy with the Ca^{2+}-ATPase, we speculated that the negatively charged Glu^{83} and the positively charged Lys^{86} and Arg^{89} in M1 of the Na^+,K^+-ATPase are located in close relation to the cytoplasmic inlet for Na^+, where they might contribute to a Na^+ selectivity filter. The mutagenesis results on the Na^+,K^+-ATPase (Einholm et al. 2007) strongly argue against any importance of the side chains of Lys^{86} and Arg^{89} for Na^+ migration, by demonstrating that individual substitutions of these residues with the smaller alanine and the oppositely charged glutamate are fully tolerated with no or only an insignificant effect on the apparent affinity for Na^+. Neither did these mutations affect the apparent affinity for K^+. Furthermore, the negatively charged Glu^{83} appeared to be without much importance for Na^+ and K^+ affinity, indicating that this residue is not part of a binding site along the cation migration pathway. Not only were the cation binding properties found unaffected by the mutations of these charged residues, but so were also the rate of the overall reaction and the conformational changes of the enzyme. Considering that Glu^{83}, Lys^{86}, and the positive charge at position 89 (either an arginine or a lysine) are highly conserved among Na^+,K^+-ATPases and H^+,K^+-ATPases, and that Glu^{51}, Glu^{55}, and Glu^{58} are all fully conserved among all SERCAs, the apparent lack of functional importance of these residues is surprising. It is possible that it is not the individual side chains, but their joint effect on polarity, that is critical. The charged/polar and hydrophobic residues tend to be distributed on opposite sides of the N-terminal part of M1 helix, making it amphipathic and in this way dictating the orientation of the bend with respect to the water–lipid interface.

Importance of hydrophobic residues in the middle part of M1, Leu^{65}/Leu^{99} is the gatekeeper

The second crystal structure of the Ca^{2+}-ATPase, the Ca^{2+}-free E_2 form with thapsigargin bound (Toyoshima and Nomura 2002), drew attention to a water-accessible channel leading between M1 and M3 to Glu^{309} at the Ca^{2+} site, which has opened by partial unfolding and bending of the helical structure of M1 at Asp^{59}. This access pathway, having the potential of a Ca^{2+} entrance, is lined on one side by hydrophobic side chains from the middle part of M1 (Leu^{60}, Leu^{61}, Val^{62}, and Leu^{65}), which encouraged us to design additional mutations in the middle part of M1 of the Ca^{2+}-ATPase (Einholm et al. 2004) and the Na^+,K^+-ATPase (Einholm et al. 2007) to investigate the demands for hydrophobicity and bulkiness of residues in the wall lining this channel.

Some of the hydrophobic residues turned out to be much more critical than the charged residues described above. Hence, Leu^{60} and Val^{62} of the Ca^{2+}-ATPase were found important for Ca^{2+} interaction, as evidenced by significantly reduced apparent affinities for Ca^{2+} activation of phosphorylation and increased rate constants for the Ca^{2+} dissociation from Ca_2E_1 in mutants with alteration to these residues (Einholm et al. 2004). One possibility for interpretation of these data is that the mutation increased the dimensions of the channel between M1 and M3 through destabilization of the wall, resulting in an easier passage of Ca^{2+}. The channel between M1 and M3 is obvious in all the E_2 crystal structures of the Ca^{2+}-ATPase, and in the E_2 crystal structures with the inhibitors 2,5-di-*tert*-butyl-1,4-dihydroxybenzene (BHQ) and cyclopiazonic acid (CPA). These inhibitors are bound right in the cytoplasmic mouth of the channel, interacting with Asp^{59}, Leu^{61}, Val^{62}, and Leu^{65} and probably blocking Ca^{2+} entry (Obara et al. 2005; Moncoq et al. 2007; Takahashi et al. 2007).

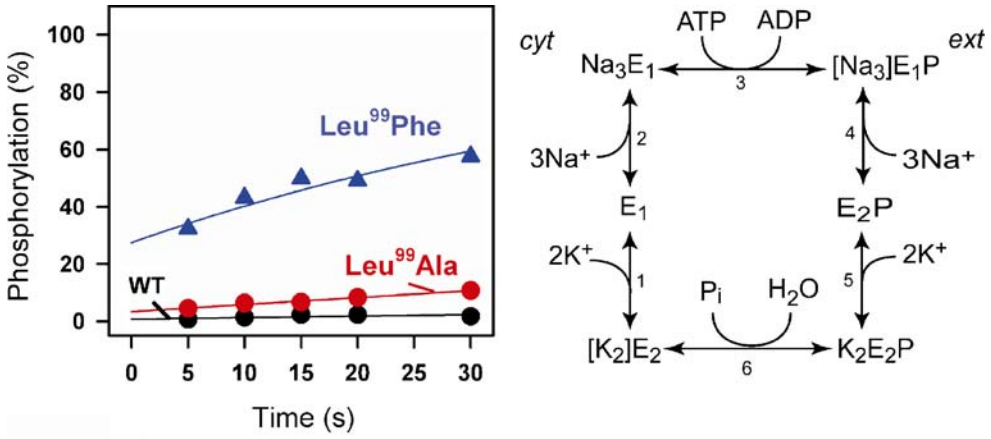
Of the M1 residues substituted in the Ca^{2+}-ATPase, Leu^{65}, which is located at the bottom of the above discussed channel, is most important to Ca^{2+} occlusion. The occlusion gate seems more stably closed following reduction of the size of the hydrophobic side chain of Leu^{65}, as substitution of Leu^{65} with alanine led to a dramatic 11-fold decrease in the rate of Ca^{2+} dissociation from the unphosphorylated E_1 form. Furthermore, the $E_1P \rightarrow E_2P$ step was found 4-fold faster in this mutant relative to the wild type, and the dephosphorylation of E_2P was blocked. The fact that this single mutation reduces the rate of Ca^{2+} dissociation from E_1 as much as 11-fold provides a nice illustration of the concept that even the unphosphorylated E_1 enzyme exists in an equilibrium between occluded ($[Ca_2]E_1$) and non-occluded (Ca_2E_1) states (Forbush 1988; Orlowski and Champeil 1991; Vilsen and Andersen 1992a). By stabilizing the occluded state, phosphorylation of the enzyme serves "to lock a door

that has already been closed" (Vilsen 1995b), and apparently the Leu^{65}Ala mutation has a similar effect favoring the closed state. The importance of M1 for Ca^{2+} occlusion gained further support when the atomic structure of the Ca^{2+}-ATPase in the occluded conformation was subsequently determined (Toyoshima and Mizutani 2004; Sorensen et al. 2004). In this structure, Leu^{65} is close enough to Glu^{309} of M4 to make van der Waals contact (cf. Fig. 2b), and Leu^{65} may function as "gate-locking" residue, which by interaction with the side chain of the gating residue Glu^{309} at the Ca^{2+} site restricts the mobility of the gate (Einholm et al. 2007). It can be envisaged that reduction of the size of the side chain in the Leu^{65}Ala mutant will lead to a collapse of the migration pathway, and that under these conditions the position of Glu^{309} remains even more fixed, thereby explaining the stabilization of the occluded form.

Considering the importance of Leu^{65} of the Ca^{2+}-ATPase for Ca^{2+} occlusion it was very stimulating to look at the alignment in Fig. 1, according to which this leucine is conserved between the Ca^{2+}-ATPase and the Na^+,K^+-ATPase, the corresponding residue being Leu^{99} of the Na^+,K^+-ATPase (cf. also Fig. 2d). In fact, this leucine seems to be conserved among all members of the Ca^{2+}-ATPase, the Na^+,K^+-ATPase, and the non-gastric H^+,K^+-ATPase families, underscoring its vital importance. In the gastric H^+,K^+-ATPases, the equivalent residue is a methionine, which is also hydrophobic and somewhat similar to leucine in size. Importantly, mutation of Leu^{99} of the Na^+,K^+-ATPase was found to affect K^+ interaction profoundly (Einholm et al. 2007). Four mutants were studied, Leu^{99}Ala, Leu^{99}Phe, Leu^{99}Gln, and Leu^{99}Ile, and three different kinds of effects were observed: (1) acceleration of K^+ deocclusion $[K_2]E_2 \rightarrow E_1$ combined with destabilization of $[K_2]E_2$. This was seen particularly in Leu^{99}Phe, but also to some extent in Leu^{99}Ala. (2) acceleration of K^+ deocclusion $[K_2]E_2 \rightarrow E_1$ combined with normal or even increased stability of $[K_2]E_2$. This was seen only for Leu^{99}Gln; (3) reduction of the K^+ affinity of the extracellularly facing sites on E_2P, this was seen particularly in Leu^{99}Ala and to some extent in Leu^{99}Ile, but not in Leu^{99}Phe and Leu^{99}Gln. In all the three mutants Leu^{99}Ala, Leu^{99}Phe, and Leu^{99}Gln, the rate of K^+ deocclusion was significantly enhanced, but in contrast to Leu^{99}Ala and Leu^{99}Phe the equilibrium level of $[K_2]E_2$ was not reduced in Leu^{99}Gln, indicating an enhanced rate of formation of $[K_2]E_2$. Among these three mutants, the acceleration of K^+ deocclusion was most prominent for Leu^{99}Phe, and even when Rb^+ was used as congener of K^+ to stabilize the occluded form as much as possible, the rate of Rb^+ deocclusion was increased by as much as 39-fold relative to wild type, i.e. more than seen for mutation of the K^+ binding residue Glu^{329} (Fig. 3). These findings are consistent with the hypothesis that Leu^{99} and Glu^{329} interact and cooperate in K^+ binding and gating of the K^+ sites as modeled in Fig. 2d. As a consequence of the close apposition between the side chains of Leu^{99} in M1 and Glu^{329} in M4, the replacement of Leu^{99} with the larger phenylalanine leads to steric clash with Glu^{329}, thus explaining the marked destabilization of the $[K_2]E_2$ form in Leu^{99}Phe (Einholm et al. 2007). The insertion of an alanine does not lead to steric collision with Glu^{329} and is therefore less destabilizing to $[K_2]E_2$, however, the fact that the Leu^{99}Ala mutant showed a significant 5-fold increase in the deocclusion rate (i.e. more than seen for the Leu^{99}Ile mutant), indicates that the presence of a side chain of a certain size is necessary in order to restrict the movement of the Glu^{329} side chain as required for proper K^+ occlusion.

The cells expressing Leu^{99}Ala required additional K^+ in the medium to survive, and direct measurements of K^+ activation of dephosphorylation using a rapid-mixing technique showed a most conspicuous 17-fold reduction in the affinity of E_2P for K^+ in Leu^{99}Ala, whereas Leu^{99}Phe and Leu^{99}Gln were wild type-like in this respect (cf. Fig. 4). The reduced V_{max} of the dephosphorylation reaction $K_2E_2P \rightarrow [K_2]E_2$ in Leu^{99}Phe (see table in Fig. 4) may be explained by a destabilization of the transition state similar to that seen for $[K_2]E_2$. It is noteworthy that the smaller alanine was most disturbing to the K^+ affinity of the E_2P form. The differential effects of the alanine and phenylalanine substitutions on K_2E_2P and $[K_2]E_2$ illustrate very well that these forms are structurally different in line with the assumption that in the $[K_2]E_2$ form K^+ is occluded with no access to either side of the membrane, whereas in K_2E_2P the K^+ binding site has opened up toward the extracellular surface. In the latter conformation there may be more space available around Leu^{99}, which may prevent steric clash between the phenylalanine and the glutamate, thus explaining the close to normal K^+ affinity of E_2P in Leu^{99}Phe. An explanation of the low K^+ affinity of E_2P in Leu^{99}Ala could be that bulkiness required to hold the ion binding pocket open for K^+ access from the extracellular side is lacking in this mutant (Einholm et al. 2007).

It is furthermore of note that Leu^{99}Gln was found incapable of keeping K^+ efficiently occluded, even though this mutant retained the ability to bind K^+ with a wild type-like affinity. Because both occlusion and deocclusion appeared to be accelerated in Leu^{99}Gln, it seems that the ability to keep the occlusion gate shut has been lost due to disruption of the gate-locking mechanism. In the terminology of the "flickering gate model" proposed by Forbush (Forbush 1987, 1988), this corresponds to a more frequent flickering of the gate, which might be explained by more freedom of movement of the Glu^{329} side chain (see Fig. 10 in Einholm et al. 2007, for structural details explaining that a glutamine at position 99 restricts the mobility of Glu^{329} side chain less than the leucine). The increased rate of release of

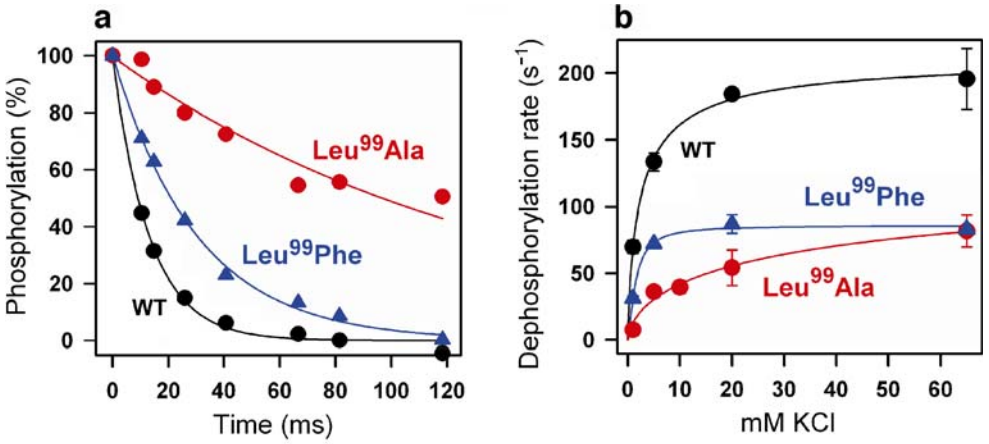


	$[Rb_2]E_2$ (%)	$[Rb_2]E_2 \rightarrow E_1$ k_{obs}
WT	99	1
$Leu^{99}Ala$	97	5
$Leu^{99}Phe$	73	39
$Glu^{329}Gln$	87	30

Fig. 3 The potassium/rubidium occluded E_2 form of the Na^+,K^+-ATPase is severely destabilized by replacement of Leu^{99} with phenylalanine. To study the occlusion and deocclusion of Rb^+ as a K^+ congener, the enzyme was equilibrated with Rb^+ in the absence of Na^+ and ATP, and the time course of phosphoenzyme formation was subsequently monitored upon dilution of the enzyme in a solution containing [γ-^{32}P]ATP and Na^+ required for phosphorylation (Einholm et al. 2007). For the Rb^+-occluded enzyme, phosphorylation proceeds through the deocclusion of Rb^+ (step 1 in the scheme), the binding of Na^+ to the E_1 form (step 2) and the reaction with ATP (step 3), where step 1 is rate limiting. It is seen that in particular the $Leu^{99}Phe$ mutant deviates from the wild type in two aspects. First, the intercept with the ordinate is higher in the mutant, reflecting that a higher fraction of enzyme binds Na^+ instantaneously, because it does not contain occluded Rb^+. Hence, the equilibrium level of Rb^+-occluded enzyme is decreased in the mutant (to 73 versus 99% in wild type, see the table). Secondly, the rate constant for deocclusion of Rb^+ is much higher in the mutant compared with wild type, as indicated by the slope of the line, corresponding to a 39-fold increase in the rate constant for the $Leu^{99}Phe$ mutant, relative to the wild type (see the table). The destabilization of the Rb^+-occluded E_2 form in $Leu^{99}Phe$ is even more pronounced than seen for mutation of the cation binding Glu^{329} in M4

occluded K^+ from $[K_2]E_2$ does not allow a distinction between whether it is the cytoplasmic gate or the extracellular gate that is affected. It could be both. Relative to E_1, Glu^{329} moves closer to the extracellular surface in E_2/E_2P (Vilsen and Andersen 1998) and could form an extracellular gate in these states. Mutation $Leu^{99}Gln$ furthermore affected the ability of the Na^+,K^+-ATPase to distinguish between Na^+ and K^+ at the extracellularly facing binding sites of E_2P, as indicated by an improved ability of Na^+ to act as surrogate for K^+ in activation of dephosphorylation, thus resulting in extraordinary Na^+-ATPase activity (Einholm et al. 2007). Apparently, a hydrophobic residue at the position of Leu^{99} prevents Na^+, but not K^+, from being an efficient activator of E_2P dephosphorylation, whereas the glutamine promotes Na^+ activation of the E_2P dephosphorylation.

When the conspicuous effects of the Leu^{99} mutations on K^+ occlusion and K^+ affinity of the extracellularly facing sites are compared with our observations with the Ca^{2+}-ATPase, it should be borne in mind that a role for proton binding in activation of dephosphorylation of Ca^{2+}-ATPase analogous to that for K^+ in connection with Na^+,K^+-ATPase dephosphorylation is difficult to demonstrate directly, because pH changes exert multiple functional effects. The Na^+,K^+-ATPase is therefore an advantageous experimental system compared with the Ca^{2+}-ATPase for characterization of the interaction with counterions. Nevertheless, the block of E_2P dephosphorylation observed in the Ca^{2+}-ATPase mutant $Leu^{65}Ala$ is strongly reminiscent of the markedly reduced affinity of the extracellularly facing sites of E_2P for K^+ in the Na^+,K^+-ATPase mutant $Leu^{99}Ala$, thus suggesting that also in the Ca^{2+}-ATPase the leucine is important for the interaction with the extracytoplasmic counterions. Another similarity between the Ca^{2+}-ATPase mutant $Leu^{65}Ala$ and the Na^+,K^+-ATPase mutant $Leu^{99}Ala$ is the acceleration of the $E_1P \rightarrow E_2P$ conformational transition, which is intimately associated with Na^+/Ca^{2+} translocation across the membrane. It seems likely that this acceleration arises from an increased rate of deocclusion of the Na^+/Ca^{2+} ions bound in E_1P, which may be accounted for by a relief of the conformational constraint on the Glu^{329} side chain caused



	$K_{0.5}$ (K^+)	V_{max}
WT	1	1
$Leu^{99}Ala$	17	0.7
$Leu^{99}Phe$	0.6	0.4
$Glu^{329}Gln$	9	0.9

Fig. 4 The apparent affinity of the E_2P form of the Na^+,K^+-ATPase for extracellular K^+ is severely reduced by replacement of Leu^{99} with alanine. To study the K^+ dependence of dephosphorylation of E_2P (step 6 in the scheme of Fig. 3), the latter intermediate was formed by phosphorylation with [γ-^{32}P]ATP in the presence of Na^+, and dephosphorylation was followed upon addition of an excess of unlabeled ATP and various concentrations of K^+ (Einholm et al. 2007). **a** The time course of dephosphorylation of E_2P in the presence of 1 mM K^+. **b** K^+ dependence of rate constants determined as in (**a**). The affinity for extracellular K^+ is severely reduced in the $Leu^{99}Ala$ mutant corresponding to as much as 17-fold, which is even more than seen for mutation of the cation binding Glu^{329} in M4. The $Leu^{99}Phe$ mutation did not reduce the K^+ affinity of E_2P, but reduced the V_{max} of the E_2P dephosphorylation reaction, probably due to destabilization of the transition state of the reaction, where the ions likely have become occluded. The reduced affinity in the alanine mutant indicates that a side chain of a certain size is required for receiving the K^+ ions when they bind from the extracellular side (step 5 in Fig. 3) just as Glu^{329} in M4 is required for this binding process

by the Leu^{65}/Leu^{99} side chain, i.e. the presence of an alanine would allow the extracytoplasmic occlusion gate to flicker open more often during the transit from E_1P to E_2P. While these similarities between the functions of the leucine in the Ca^{2+}-ATPase and the Na^+,K^+-ATPase point to similar roles of M1 in the two enzymes, an important difference also appeared from our results. Hence, in the Ca^{2+}-ATPase the interaction of the E_1 form with Ca^{2+} was clearly defective in $Leu^{65}Ala$ as evidenced by the 11-fold reduction in the rate of Ca^{2+} dissociation from E_1 discussed above, whereas we obtained no evidence for a change in Na^+ interaction of the E_1 form of the Na^+,K^+-ATPase.

A pivot for movement of M1

The M1 segment of the Na^+,K^+-ATPase contains two juxtaposed glycines, Gly^{93} and Gly^{94}, located at the positions corresponding to Asp^{59} and Leu^{60} of the Ca^{2+}-ATPase (Fig. 1), i.e. right at the point, where M1 bends (Fig. 2d). In studies of the $Gly^{93}Ala$ and $Gly^{94}Ala$ mutants, evidence was provided for a critical role of Gly^{94} in the interaction with Na^+ and K^+ (Einholm et al. 2005). First of all, rapid kinetic measurements of the K^+ dependence of E_2P dephosphorylation revealed a remarkable 9-fold reduction of the K^+ affinity of the extracellularly facing sites of E_2P in mutant $Gly^{94}Ala$. Secondly, the sensitivity of the $E_1P \leftrightarrow E_2P$ equilibrium to Na^+ was found considerably reduced, with accumulation of E_2P even at a high Na^+ concentration of 600 mM where the equilibrium is shifted toward E_1P in the wild type. This finding indicates that the interaction of E_2P with extracellular Na^+ is impaired. Moreover, the ability of Na^+ to induce E_2P dephosphorylation, by acting as a substitute for K^+, was reduced in $Gly^{94}Ala$. Considering the low affinity of E_2P for K^+ in this mutant, the mutational effect on the interaction of E_2P with extracellular Na^+ presumably results from a disruption of the extracellularly facing cation binding sites of E_2P. These effects resemble those observed for mutation $Leu^{99}Ala$, but in addition $Gly^{94}Ala$ displayed a 3-fold reduced apparent affinity for Na^+ activation of phosphorylation. This was shown to be an effect on the Na^+ binding E_1 form and not due to a shift of the conformational equilibrium of the dephosphoenzyme away from E_1. Compared to $Gly^{94}Ala$,

Gly^{93}Ala was much more wild type-like with the K^+ affinity of E_2P being slightly increased rather than decreased (Einholm et al. 2005).

In the Ca^{2+}-ATPase crystal structures Ca_2E_1PADP and E_2MgF_4 (Fig. 2b and c), Leu^{60} in M1 and Gly^{257} in M3 are in rather close proximity. Metal-catalyzed oxidative cleavage studies of the Na^+,K^+-ATPase (Tal et al. 2001) furthermore seem to support the existence of a contact point between M1 and M3 in the native non-crystalline enzyme. In the Na^+,K^+-ATPase, the residues present at the positions equivalent to Leu^{60} and Gly^{257} of the Ca^{2+}-ATPase are Gly^{94} and Ile^{287}, respectively (Fig. 2d). The pair of a bulky residue and a small glycine is conserved in as well the SERCA family of ATPases as in all Na^+,K^+-ATPase isoforms across species, probably to avoid a steric clash. Hence, the bulkiness of the isoleucine/leucine probably demands that only a small glycine is present at position 94 of M1 in the Na^+,K^+-ATPase and at position 257 of M3 in the Ca^{2+}-ATPase (see Fig. 2). The introduction of the larger alanine in Na^+,K^+-ATPase mutant Gly^{94}Ala likely results in a clash with Ile^{287} that hinders the movements of M1 and/or alters the positioning of M1. This would be expected to have a direct impact on cation binding, because Leu^{99} immediately below the bending point of M1 makes van der Waals contact with the cation binding glutamate in M4 as discussed above (Fig. 2d, and Einholm et al. 2007). The fact that the Gly^{94}Ala mutation caused a 9-fold reduction of the affinity of the E_2P form for K^+, but a less conspicuous 3-fold reduction of the affinity of the E_1 form for Na^+ might be explained by the particularly short distance between the residues constituting the contact point in the E_2 structures. Thus, the Gly^{94}-Ile^{287} pair could function as a pivot, i.e. a contact point enabling the movements of M1 required for opening of the external access pathway in E_2P, in that way being critical to the accessibility of extracellular K^+ ions to their membranous binding sites. The data obtained with the Ca^{2+}-ATPase mutants Asp^{59}Arg/Leu and Leu^{60}Ala/Pro, showing effects on Ca^{2+} interaction in E_1 as well as on the rate of dephosphorylation of E_2P and an enhanced rate of $E_1P \rightarrow E_2P$ in Asp^{59}Leu (Einholm et al. 2004), may be interpreted analogously in terms of a role for these two residues right at the bending point of M1 in control of the movements of M1 important for interaction with Ca^{2+} and countertransported protons, Leu^{60} forming a pivot together with Gly^{257} of M3 as suggested above.

Conclusion

In conclusion, the picture that emerges is that the hydrophobic, middle part of transmembrane segment M1 plays a similar crucial role in Ca^{2+} interaction/occlusion in Ca^{2+}-ATPase and K^+ interaction/occlusion in Na^+,K^+-ATPase, and likely participates in the formation of a pivot for movement of M1 in relation to the cation translocation. Thus, despite the poor sequence conservation in this region, the exception being the gatekeeper Leu^{65}/Leu^{99}, a similar concept of movements of M1 in relation to the cation translocating steps applies to the Ca^{2+}-ATPase and the Na^+,K^+-ATPase and, possibly, to the whole family of P-type ATPases.

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References

Andersen JP, Vilsen B (1994) J Biol Chem 269:15931–15936
Arguello JM, Kaplan JH (1994) J Biol Chem 269:6892–6899
Askew GR, Lingrel JB (1994) J Biol Chem 269:24120–24126
Blostein R, Wilczynska A, Karlish SJ, Arguello JM, Lingrel JB (1997) J Biol Chem 272:24987–24993
Canessa CM, Horisberger JD, Louvard D, Rossier BC (1992) EMBO J 11:1681–1687
Clarke DM, Loo TW, Inesi G, MacLennan DH (1989a) Nature 339: 476–478
Clarke DM, Maruyama K, Loo TW, Leberer E, Inesi G, MacLennan DH (1989b) J Biol Chem 264:11246–11251
Clarke DM, Loo TW, MacLennan DH (1990) J Biol Chem 265:6262–6267
Clausen JD, Vilsen B, McIntosh DB, Einholm AP, Andersen JP (2004) Proc Natl Acad Sci U S A 101:2776–2781
Einholm AP, Vilsen B, Andersen JP (2003) Ana N Y Acad Sci 986: 310–311
Einholm AP, Vilsen B, Andersen JP (2004) J Biol Chem 279:15888–15896
Einholm AP, Toustrup-Jensen M, Andersen JP, Vilsen B (2005) Proc Natl Acad Sci U S A 102:11254–11259
Einholm AP, Andersen JP, Vilsen B (2007) J Biol Chem 282:23854–23866
Forbush B III (1987) J Biol Chem 262:11116–11127
Forbush B III (1988) Prog Clin Biol Res 268A:229–248
Glynn IM (1993) J Physiol 462:1–30
Hu YK, Kaplan JH (2000) J Biol Chem 275:19185–19191
Jensen AM, Sorensen TL, Olesen C, Moller JV, Nissen P (2006) EMBO J 25:2305–2314
Jewell-Motz EA, Lingrel JB (1993) Biochemistry 32:13523–13530
Kuntzweiler TA, Arguello JM, Lingrel JB (1996) J Biol Chem 271:29682–29687
Lee AG, East JM (2001) Biochem J 356:665–683
Lipkind GM, Fozzard HA (2000) Biochemistry 39:8161–8170
Lipkind GM, Fozzard HA (2001) Biochemistry 40:6786–6794
MacLennan DH, Rice WJ, Green NM (1997) J Biol Chem 272: 28815–28818
Moncoq K, Trieber CA, Young, HS (2007) J Biol Chem 282:9748–9757
Montigny C, Jaxel C, Shainskaya A, Vinh J, Labas V, Moller JV, Karlish SJ, le Maire M (2004) J Biol Chem 279:43971–43981
Obara K, Miyashita N, Xu C, Toyoshima I, Sugita Y, Inesi G, Toyoshima C (2005) Proc Natl Acad Sci U S A 102:14489–14496
Ogawa H, Toyoshima C (2002) Proc Natl Acad Sci U S A 99:15977–15982
Ogawa H, Stokes DL, Sasabe H, Toyoshima C (1998) Biophys J 75: 41–52
Olesen C, Sorensen TL, Nielsen RC, Moller JV, Nissen P (2004) Science 306:2251–2255
Orlowski S, Champeil P (1991) Biochemistry 30:352–361

Pedersen PL, Carafoli E (1987) Trends Biochemic Sci 12:146–150
Pedersen PA, Nielsen JM, Rasmussen JH, Jorgensen PL (1998) Biochemistry 37:17818–17827
Post RL, Hegyvary C, Kume S (1972) J Biol Chem 247:6530–6540
Price EM, Lingrel JB (1988) Biochemistry 27:8400–8408
Price EM, Rice DA, Lingrel JB (1989) J Biol Chem 264:21902–21906
Price EM, Rice DA, Lingrel JB (1990) J Biol Chem 265:6638–6641
Ruiz-Perez VL, Carter SA, Healy E, Todd C, Rees JL, Steijlen PM, Carmichael AJ, Lewis HM, Hohl D, Itin P, Vahlquist A, Gobello T, Mazzanti C, Reggazini R, Nagy G, Munro CS, Strachan T (1999) Hum Mol Genet 8:1621–1630
Schultheis PJ, Lingrel JB (1993) Biochemistry 32:544–550
Sorensen TL, Moller JV, Nissen P (2004) Science 304:1672–1675
Sugita Y, Miyashita N, Ikeguchi M, Kidera A, Toyoshima C (2005) J Am Chem Soc 127:6150–6151
Sweadner KJ, Donnet C (2001) Biochem J 356:685–704
Takahashi M, Kondou Y, Toyoshima C (2007) Proc Natl Acad Sci U S A 104:5800–5805
Tal DM, Capasso JM, Munson K, Karlish SJ (2001) Biochemistry 40:12505–12514
Toyoshima C, Mizutani T (2004) Nature 430:529–535
Toyoshima C, Nomura H (2002) Nature 418:605–611
Toyoshima C, Nakasako M, Nomura H, Ogawa H (2000) Nature 405: 647–655
Toyoshima C, Nomura H, Tsuda T (2004) Nature 432:361–368
Vilsen B (1995a) Biochemistry 34:1455–1463
Vilsen B (1995b) Acta Physiol Scand Suppl 624:1–146
Vilsen B, Andersen, JP (1992a) J Biol Chem 267:3539–3550
Vilsen B, Andersen JP (1992b) J Biol Chem 267:25739–25743
Vilsen B, Andersen JP (1998) Biochemistry 37:10961–10971
Vilsen B, Andersen JP, Petersen J, Jorgensen PL (1987) J Biol Chem 262:10511–10517