Review Article

Structural Biology of the Sodium Pump

P-type ATPases are ATP-powered ion pumps, and include Ca^{2+}-ATPase, Na^+,K^+-ATPase, among others. Best understood structurally and biochemically is Ca^{2+}-ATPase from sarcoplasmic reticulum of fast twitch skeletal muscle (sarco(endo) plasmic reticulum Ca^{2+}-ATPase 1a, SERCA1a). Since our publication of the first crystal structure of SERCA1a in 2000, which was the first ion pump structure determined at near atomic resolution, the pump has been a continuous source of excitement, as crystal structures of new reaction intermediates always show large and unexpected structural changes. In the P-type ATPase reaction cycle, there are two major states, E1 and E2 [1] (Fig. 1(a)). The E1 state has high affinity for cations to be transferred from the cytoplasm into the extracellular medium (or lumen). In this state, SERCA1a binds two Ca^{2+} with high affinity, whereas Na^+,K^+-ATPase bind three Na^+. In the E2 state, the transmembrane cation binding sites bind these ions with low affinity and counter ions with high affinity. That is, SERCA1a binds 2 or 3 H^+ with high affinity, whereas Na^+,K^+-ATPase binds two K^+. The crystal structures show that very large rearrangements of three cytoplasmic domains and 10 transmembrane helices take place during the reaction cycle. A 110° rotation of the A-domain accompanying the transition from E1P → E2·Pi is one of the largest domain movements ever observed as far as the authors are aware (Fig. 1(b)). For SERCA1a, crystal structures have been determined using BL41XU for most of the reaction intermediates, nearly covering the entire reaction cycle. We can now describe a quite detailed scenario for the active transport of Ca^{2+} by this ATPase [1]. In 2013, we published a long awaited E1 structure with and without sarcolipin [2], a regulatory protein in skeletal and heart atrial muscles. As we have already published a review article [3] on this crystal structure, here we focus on our other target, the Na^+,K^+-ATPase, which, as we have described, transports three Na^+ out and two K^+ into the cytoplasm per molecule of ATP hydrolysed (Fig. 1(a)). Na^+,K^+-ATPase is expressed in all animal cells and plays critical roles in many fundamental processes in life, notably excitation of nerve cells and

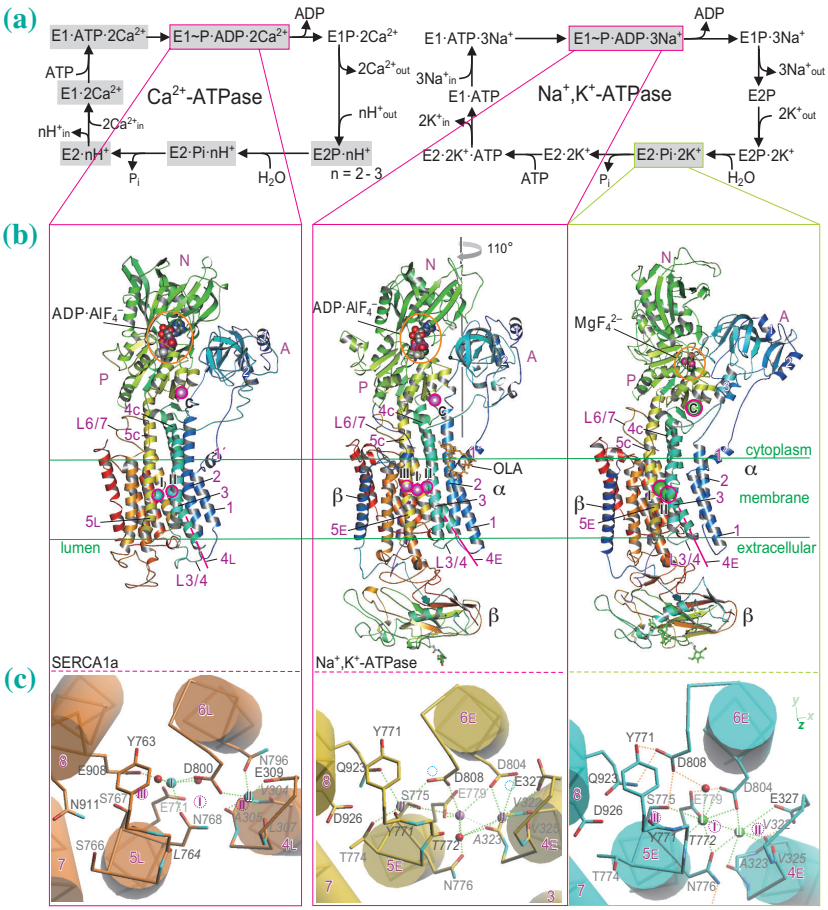

Fig. 1. **(a)** The reaction cycle and crystal structures of Na^+,K^+-ATPase in comparison with those of SERCA1a. The intermediates of which crystal structures have been published are shaded (top panel). **(b)** The Na^+ (or K^+) under the P-domain (c) has a regulatory role. **(c)** The bottom panels show the transmembrane cation binding sites viewed from the cytoplasm. Small violet spheres represent Na^+, cyan ones Ca^{2+}, green ones K^+ and red ones water. Dotted circles in cyan indicate the positions of bound Ca^{2+} in SERCA1a and purple ones bound Na^+ in Na^+,K^+-ATPase.

contractions of heart muscle. As a consequence, Na^+,K^+-ATPase is implicated in many diseases, such as high blood pressure, neurological disorders and cancers. It is the target of digitalis-like compounds (now called cardiotonic steroids (CTS)), which have been prescribed for more than two centuries for treatment of heart failure and arrhythmias. Furthermore, Na^+,K^+-ATPase may function as a docking station, regulated by endogenous CTS. In fact, a number of proteins have been reported to interact with Na^+,K^+-ATPase. Since Na^+,K^+-ATPase is up regulated in certain cancer types, it has been gathering attention as a new anti-tumor target [4].

Compared to Ca^{2+}-ATPase, structural elucidation of Na^+,K^+-ATPase lags far behind. Na^+,K^+-ATPase is a substantially more complex pump than SERCA1a, consisting of α and β subunits and an auxiliary FXYD protein. The α-subunit is the catalytic subunit, with a very similar architecture to SERCA1a, has four distinct domains: cytoplasmic actuator (A), phosphorylation (P) and nucleotide binding (N) domains and a transmembrane domain. The β-subunit is considered to be the molecular chaperone of the α-subunit and is heavily glycosylated; this subunit is unique to Na^+,K^+-ATPase and H^+,K^+-ATPase. The FXYD protein is a tissue specific regulator for fine tuning of catalytic activity and stability (Fig. 1(b)). The first crystal structure of this ATPase was published in 2007 by Morth *et al.* [5]. It showed an E2·2K^+·Pi state at only 3.5 Å resolution and lacked information on most of the β-subunit. We published a 2.4 Å resolution structure for the same state two years later [6] and provided a description of nearly the entire pump, including details of K^+-coordination. This crystal structure gave answers to several fundamental questions, such as, why Na^+,K^+-ATPase countertransports K^+ whereas Ca^{2+}-ATPase does not, even though almost the same residues are present in the binding sites (reviewed in [7]).

Crystal structure of Na^+, K^+-ATPase in the E1~P·ADP·3Na^+ state

Nevertheless, Na^+-bound structures are far more important, because Na^+,K^+-ATPase is essentially a Na^+-pump: only Li^+ and H^+ can partially substitute for Na^+, but K^+ can be replaced with many monovalent cations, including even Na^+, and organic cations. Of particular interest is where the third Na^+ site, specific to Na^+,K^+-ATPase is located. Furthermore, the affinity of Na^+,K^+-ATPase for Na^+ is very low (~ mM K_d; 15 mM from charge transfer), compared to that of SERCA1a for Ca^{2+} (μM to sub-μM). K^+ has a higher affinity and converts the ATPase into the E2 state. Yet, Na^+,K^+-ATPase strictly transfers only Na^+, rejecting K^+ and Ca^{2+}, in the forward direction. The turnover number of the ATPase is even larger than that of SERCA1a. Explanations about these differences awaited crystal structures of the E1 species with bound Na^+. We recently succeeded in determining crystal structures of this ATPase in E1·AlF_4-·ADP with three bound Na^+ at 2.8 Å resolution using BL41XU [8]. This structure is thought to represent the transition state (E1~P·ADP·3Na^+) that precedes the occluded E1P[3Na^+] state. We chose this state, as the cytoplasmic gate is at a closed position, with 3Na^+ already occupying the transmembrane cation binding sites, to evade problems that arise from the low affinity for Na^+.

The crystal structures show three Na^+ bound in the transmembrane region (Fig. 1(b,c)) and exhibit several distinct structural features. It is understandable that K^+ has a high affinity for the Na^+-binding sites, as thermal movements will slightly alter the conformations of the coordinating side chains allowing larger ions to bind. In fact, site I appears large enough to accommodate K^+ (Fig. 2). To minimise such movements, three strategies appear to be used. The first one is the use of main chain carbonyls for coordination. Indeed, the Na^+-binding sites are highly offset to the M5-side compared to the Ca^{2+}-binding sites in SERCA1a for this purpose, although the coordinating residues are almost identical to those in SERCA1a (Fig. 1(c)). The second is the

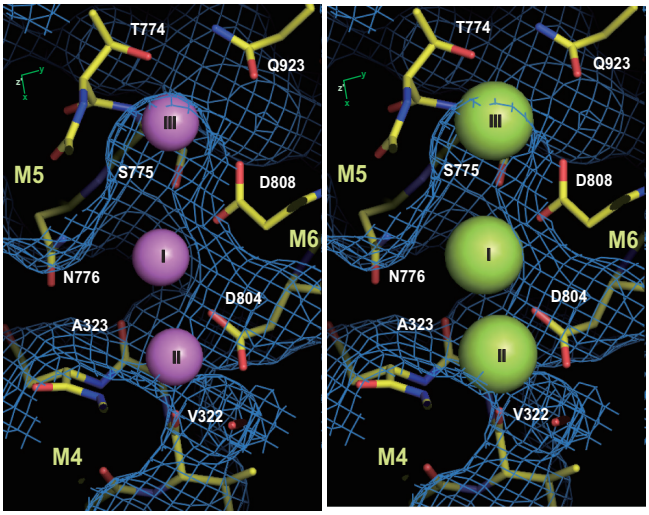

Fig. 2. Continuous cavity connecting three Na^+-binding sites. Na^+-accessible surface (blue nets) with three Na^+ (violet spheres) and K^+ (green spheres) placed in the Na^+-binding sites.

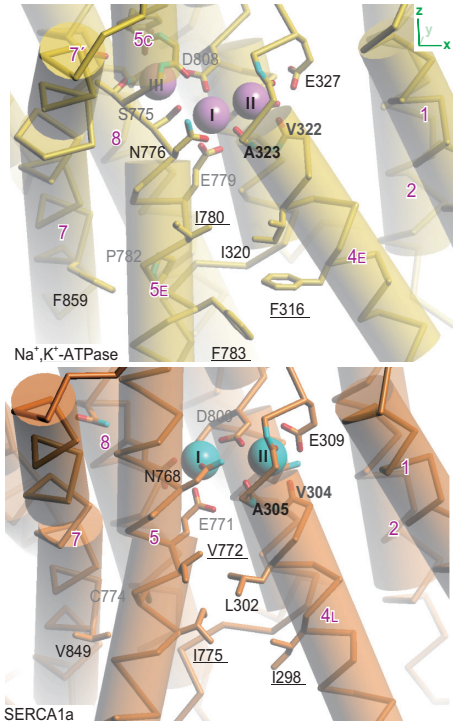

Fig. 3. Enlarged views of the transmembrane region of Na^+,K^+-ATPase (yellow) and SERCA1a (orange) around the cation-binding sites. Viewed approximately parallel to the membrane. M5 consists of two halves (M5C and M5E) in Na^+,K^+-ATPase but is contiguous in SERCA1a. Residue names with underscores indicate that they are substituted with bulkier residues in Na^+,K^+-ATPase.

sharing of a carboxyl group by two adjacent sites. The bindings of the three Na^+ are all sequential and co-operative. The binding of the first Na^+ to site III creates site I. Sites III and I share the carboxyl group of Asp808 and sites I and II share that of Asp804. Coordination of Na^+ will fix the orientation of the carboxyl group in a position suitable for binding of the right-sized ion (i.e. Na^+) but not larger ions (i.e. K^+). In this way, thermal movements of the coordinating residues can be effectively reduced. The third is the juxtaposition of the transported ions. The very short distance (3.4 Å) between Na^+ at sites I and II indicates that larger K^+ or divalent cations (e.g. Ca^{2+}) cannot bind because of the physical size and/or electrostatic repulsion (Fig. 2). This is achieved by bringing site II closer to site I by tilting the extracellular half of M4 (M4E) larger than in SERCA1a (Fig. 3). As a result, the main chain carbonyl of Ala323, corresponding to Ala305 dedicated to site II in SERCA1a, now contributes to site I in Na^+,K^+-ATPase. A large space formed between M4E and M5 should be filled with bulkier hydrophobic residues. Indeed, Ile and Val in SERCA1a are replaced by Phe and Leu in Na^+,K^+-ATPase (Fig. 3).

Another conspicuous strategy is that only the binding of the right-sized ion (i.e. Na^+) to site III allows productive phosphoryl transfer from ATP. Site III is highly confined so as to accommodate only Na^+ (ionic radius = 0.95 Å) and not K^+ (1.33 Å) (Fig. 2). However, as it is located in the hinge between the two halves of the M5 helix (M5C and M5E) (Fig. 3), K^+ appears to be able to bind if M5C moves 10° or so towards M7. As M5C is integrated into the P-domain (Fig. 1(b)), it controls the position of the P-domain. That is, for phosphoryl transfer from ATP to the invariant Asp residue in the P-domain to take place, a proper arrangement of the three cytoplasmic domains is necessary. Such an arrangement is realised only by the binding of the right sized ion (i.e. Na^+) to site III. It should be noted that the orientation of M5C also determines the inclination of M4E, through the L6/7 loop and the M3 helix.

Thus, we now see the really intricate maneuvers that Na^+,K^+-ATPase uses for selecting Na^+. In view of this, the Ca^{2+} binding sites in SERCA1a appear very primitive. Yet, SERCA1a rigorously selects Ca^{2+}, even in a milieu of 10,000 times more concentrated Na^+, which has a very similar ionic radius (0.95 *vs* 0.99 Å). This will mean that charge balance is critical in maintaining the binding sites with right geometries or in causing proper structural changes for creating the binding sites.

Visualization of the substitution process of K^+ bound to Na^+,K^+-ATPase

It is well established that two K^+ bind sequentially to the empty cation binding sites after the release of three Na^+ into the extracellular medium (Fig. 1(c)), but to which site the first K^+ binds was unknown. It is also well established that the two bound K^+ can be substituted with K^+ congeners in two (fast and slow) phases in the presence of Pi, and only a half of the bound K^+ is substituted in the fast phase. To explain these experimental findings, Forbush proposed a "flickering gate model" [9], in which only K^+ at the "fast" site can be substituted with congeners, such as Rb^+ and Tl^+. Such a sequential binding/release model implies that the "fast" site must be the site for the last (i.e. second) K^+ to bind. Conventional kinetics experiments using radio isotopes cannot answer which of the two binding sites is the "fast" site, but X-ray crystallography could, using Tl^+ or Rb^+ as the substituent, because they have larger atomic numbers and significant anomalous scattering cross-sections. Indeed,

Fourier difference and anomalous difference maps calculated from diffraction data collected at BL41XU from the crystals of Na^+,K^+-ATPase in $E2{\cdot}MgF_4^{2-}{\cdot}2K^+$ (a stable analogue of $E2{\cdot}Pi{\cdot}2K^+$) soaked in a buffer containing either Tl^+ or Rb^+, showed clear peaks at the K^+-binding sites (Fig. 4(a)) [10]. By changing the time of incubation, we clearly showed that site II is the "fast" site. Thus, we have demonstrated that X-ray crystallography can provide spatial resolution to kinetics measurements.

Then the obvious question to be addressed is how these ions are gated. We have already elucidated the gating mechanism of SERCA1a for Ca^{2+} in the E1 state, but it could be entirely different for the counter ions in the E2 state. In the case of Na^+,K^+-ATPase, there may be different gating mechanisms for Na^+ and K^+ on the cytoplasmic and extracellular sides respectively. We would like to know the movements of the gating segment and the residues participating. With crystals of Na^+,K^+-ATPase in $E2{\cdot}MgF_4^{2-}{\cdot}2K^+$, such information can be obtained by analysing the temperature factors of all atoms of the protein at once. This is because thermal movements of an atom can be separated into two components: one from anisotropic collective movements of a segment (e.g. an α-helix) on which that particular atom is located, and the other from random isotropic movements of that atom. The answer is again quite clear: the M3 and M4 helices move together and work as a swing door to the ion pathway (Fig. 4(b,c)). The movements predicted by the temperature factor analysis were very similar to those caused by ouabain binding to the $E2{\cdot}MgF_4^{2-}{\cdot}2K^+$ crystal [11], indicating that such movements are indeed possible. Thus, we demonstrated that it is possible to predict how a gate will open from a crystal structure with the gate closed.

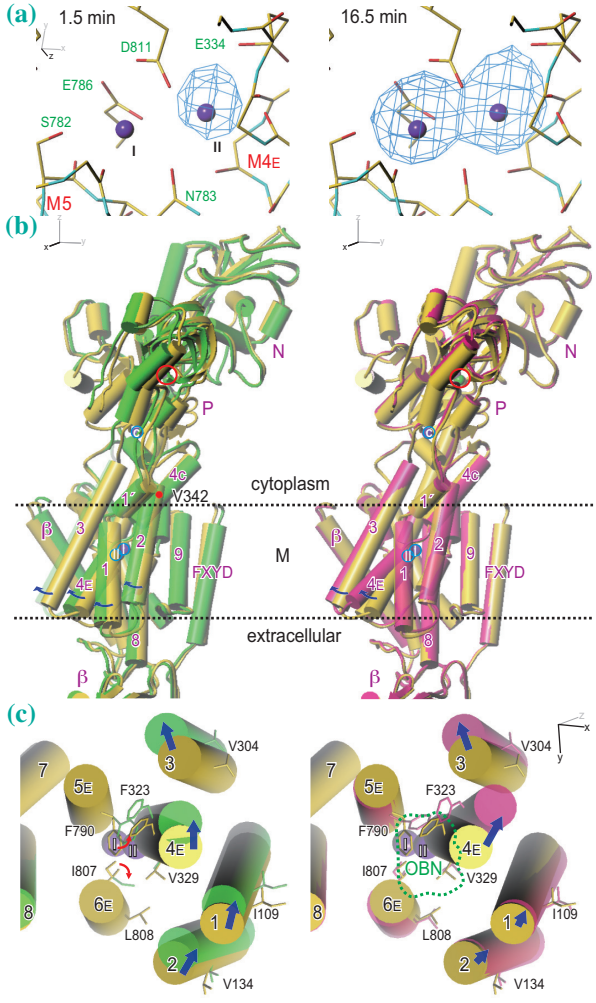

Fig. 4. Sequential substitution of bound K^+ with Tl^+ and segmental movements of Na^+,K^+-ATPase in the $E2{\cdot}2K^+{\cdot}MgF_4^{2-}$ crystal. **(a)** $|F_{obs}(Tl^+)| - |F_{obs}(K^+)|$ difference Fourier maps from crystals soaked in a buffer containing Tl^+ for 1.5 min and 16.5 min. **(b)** and **(c)** Superimpositions of the atomic models of the ATPase in the 'average' (yellow) and 'open' (light green) or ouabain-bound (pink) forms. Viewed from the M1 to M10 direction (b) or from the extracellular side (c). Small red arrows in (c) indicate likely conformation changes of the side chains to open the ion pathway.

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References

[1] C. Toyoshima: Arch Biochem Biophys. **476** (2008) 3.
[2] C. Toyoshima *et al.*: Nature **495** (2013)260.
[3] C. Toyoshima and F. Cornelius: Curr. Opinion Struct. Biol. **23** (2013) 507.
[4] I. Prassas, E.P. Diamandis: Nat. Rev. Drug Discov. **7** (2008)926.
[5] J.P. Morth *et al.*: Nature **450** (2007) 1043.
[6] T. Shinoda *et al.*: Nature **459** (2009) 446.
[7] C. Toyoshima *et al.*: Structure **19** (2011)1732.
[8] R. Kanai *et al.*: Nature **502** (2013) 201.
[9] B. Forbush *et al.*: J. Biol. Chem. **262** (1987) 11116.
[10] H. Ogawa, F. Cornelius, A. Hirata and C. Toyoshima: Nat. Commun. **6** (2015) 8004.
[11] H. Ogawa *et al.*: Proc. Natl. Acad. Sci. USA **106** (2009) 13742.