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Review

How Ca^{2+}-ATPase pumps ions across the sarcoplasmic reticulum membrane

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ABSTRACT

Ca^{2+}-ATPase of muscle sarcoplasmic reticulum is an ATP-powered Ca^{2+}-pump that establishes a >10,000 fold concentration gradient across the membrane. Its crystal structures have been determined for 9 different states that cover nearly the entire reaction cycle. Presented here is a brief structural account of the ion pumping process, with emphasis on why the structure has to be so.

Ca^{2+}-ATPase of skeletal muscle sarcoplasmic reticulum (SERCA1a), an integral membrane protein of M_r 110K, is an ATP-powered Ca^{2+}-pump and comprises a single polypeptide chain of 994 amino acid residues (Fig. 1a). It was first identified in the 'relaxing factor' of muscle contraction and gave rise to the calcium theory that Ca^{2+} is a fundamental and ubiquitous factor in the regulation of intracellular processes [1]. In muscle contraction, Ca^{2+} is released from sarcoplasmic reticulum (SR) into muscle cells via Ca^{2+}-release channel. Ca^{2+}-ATPase then pumps back the released Ca^{2+} into the SR to cause relaxation. This pump runs as long as ATP and Ca^{2+} are present in the cytoplasm, and establishes a $>10^4$-fold concentration gradient across membranes. SERCA1a is both structurally and functionally the best characterised member of the P-type (or E1/E2-type) ion translocating ATPases. According to the classical E1/E2 theory [2,3], transmembrane Ca^{2+}-binding sites have high affinity and face the cytoplasm in E1, and have low affinity and face the lumen of SR (or extracellular side) in E2. Actual transfer of bound Ca^{2+} is thought to take place between two phosphorylated intermediates, E1P and E2P, in exchange of H^+ from the lumenal side (Fig. 1b).

We crystallised Ca^{2+}-ATPase from rabbit white skeletal muscle (SERCA1a) in phospholipid bilayer and solved the first atomic structure (E1·2Ca^{2+} form) in 2000 [4] (Fig. 1a). More than 20 crystal structures have been reported from 3 laboratories for this ATPase in 9 different states that approximately cover the entire reaction cycle [4–11]. We do not yet have an exact analogue of E1P, but limited proteolysis [12,13] shows that its domain organisation must be very similar to those in E1·AMPPCP (analogue of E1·ATP) and E1·AlF_4^-·ADP (analogue of E1~P·ADP) crystal structures [6,9]. Thus, the entire reaction cycle can be described essentially with 4 principal structures depicted in Fig. 1b. We also carried out all-atom molecular dynamics simulations for wild type and some mutants to understand the functional roles of critical residues [14]. As a result, we can now propose a fairly detailed scenario of ion pumping and describe how the affinity of the transmembrane Ca^{2+} binding sites is altered, and how the lumenal gate is opened and closed by events that occur around the phosphorylation site more than 50 Å away. They also allow us to approach more fundamental questions like (i) what are the roles of ATP [6] and phosphorylation [7], (ii) why such large domain movements are necessary [7], and (iii) why H^+ countertransport is necessary despite that the SR membrane is leaky to H^+ [8].

As I recently published a detailed account on the structural evens that occur in Ca^{2+}-ATPase in [15], here I will focus more on the structural principle. General accounts on P-type ATPases can be found in [3] and [16]. Atomic co-ordinates of the aligned structures, movies on the structural changes during the reaction cycle and some results of molecular dynamics simulations are available in the author's home-page (http://www.iam.u-tokyo.ac.jp/StrBiol/resource/res.html). A very extensive database for mutations (http://www.fi.au.dk/jpa/smd/) is made available by J.P. Andersen.

1. Architecture of Ca^{2+}-ATPase

SERCA1a consists of 3 cytoplasmic domains (A, actuator; N, nucleotide binding; P, phosphorylation), 10 transmembrane (M1–M10) helices and small lumenal loops (Fig. 1a). As described below, the A-domain, connected to the M1–M3 helices with rather long linkers, works as the actuator of the transmembrane gating mechanism that regulates Ca^{2+} binding and release (Fig. 1b) [7,17]. Crystal structures show that the linkers are flexible [18], yet the mutagenesis

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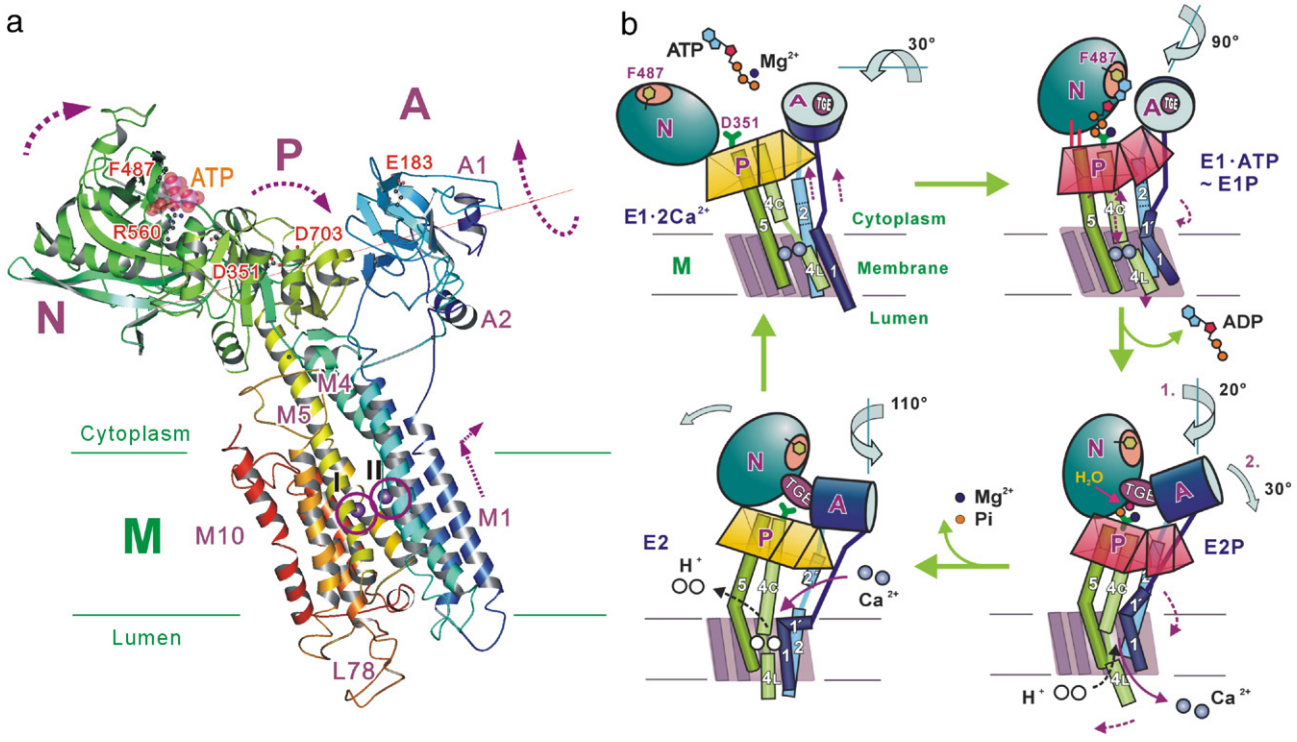

Fig. 1. Architecture of Ca^{2+}-ATPase and its ion pumping mechanism. *a*, A ribbon representation of Ca^{2+}-ATPase in the $E1\cdot 2Ca^{2+}$ state, viewed parallel to the membrane plane. Colours change gradually from the amino terminus (blue) to the carboxy terminus (red). Purple spheres (numbered and circled) represent bound Ca^{2+}. Three cytoplasmic domains (A, N and P), the α -helices in the A-domain (A1–A3) and those in the transmembrane domain (M1–M10) are indicated. M1′ is an amphipathic part of the M1 helix lying on the bilayer surface. Docked ATP is shown in transparent space fill. Several key residues—E183 (A), F487 and R560 (N, ATP binding), D351 (phosphorylation site), D627 and D703 (P) are shown in ball-and-stick. Axis of rotation (or tilt) of the A-domain is indicated with thin orange line. PDB accession code is 1SU4 ($E1\cdot 2Ca^{2+}$). *b*, A cartoon illustrating the structural changes of the Ca^{2+}-ATPase during the reaction cycle, based on the crystal structures in 7 different states. Modified from [7].

studies demonstrate that the length of the A-domain–M1 linker, at least, is critically important in gating of the ion pathway [19,20]. The A-domain contains one of the signature sequences ^{181}TGES motif [3], which plays an important role in processing of aspartylphosphate [21–23].

The P-domain contains the phosphorylation residue Asp351, Mg^{2+} co-ordinating residue Asp703 and many other critical residues that characterise the P-type ATPase as a member of the haloacid dehalogenase superfamily [24]. The P-domain is wedge shaped and has a flat top surface to allow a large rotation of the A-domain on the top surface of the P-domain [17]. The Rossmann fold of the P-domain is particularly suited for this purpose. This wedge shape is the key in converting rotational movements of the A-domain to vertical (i.e. perpendicular to the membrane) ones of the transmembrane helices. Also, the P-domain can be bent in two approximately orthogonal directions by the phosphorylation and Mg^{2+}-binding. Such flexibility is a key in realising different domain interfaces. The central β-sheet in the Rossmann fold consisting of two halves allows such bending and is also used as the secondary hinge in SERCA1a to allow an extra 30 ° not covered by the primary hinge between the N- and P-domains.

The N-domain, a long insertion between two parts of the P-domain, contains the residues (e.g. Phe487) for adenosine binding and those (e.g. Arg560) critical for bridging ATP and the P-domain [6,9] but exhibit little structural changes.

These three domains are well separated in the $E1\cdot 2Ca^{2+}$ crystal structure but gather to form a compact headpiece in the other states (Fig. 1b). The arrangement depicted in Fig. 1a may not represent the largest population in solution. Nevertheless, the amount of inclination of the N-domain with respect to the P-domain observed between $E1P\cdot$ADP and $E2\cdot$Pi analogues (60 °) is even larger, although both of them have a compact headpiece, than that between E2(TG) and $E1\cdot 2Ca^{2+}$ (50 °) [15].

There are 10 transmembrane helices and some of them (M2–M5) have long cytoplasmic extensions (Fig. 1a). In particular, M5 is 60 Å long and extends from the lumenal surface of the membrane to the end of the P-domain, working as the spine of the molecule. Two (M4 and M6) helices have proline residues in the middle and are partly unwound throughout the reaction cycle. M4–M6 and M8 contain the residues directly co-ordinating two Ca^{2+}. All helices from M1 to M6 move considerably during the reaction cycle, whereas M7–M10 helices do not. They apparently act as a membrane anchor in SERCA1a but likely to have more dynamic functional roles in Na^+K^+-ATPase [25] (T. Shinoda, H. Ogawa, F. Cornelius, and C. Toyoshima, unpublished result). In SERCA1a, the middle part of M7 containing a GXXG motif [26] makes a tight couple with M5 and allows the bending of M5.

2. Transmembrane Ca^{2+}-binding sites

It is well established that SERCA1 has two high affinity transmembrane Ca^{2+}-binding sites and the binding is co-operative [27]. The two Ca^{2+} binding sites (I and II) are located side by side near the cytoplasmic surface of the lipid bilayer (Fig. 1a), but the binding of two Ca^{2+} is sequential [28]. Site I, the binding site for the first Ca^{2+}, is located in a space surrounded by M5, M6 and M8 helices, and formed by entirely side chain oxygen atoms and two water molecules (Fig. 2). Site II is nearly 'on' the M4 helix, which is partly unwound and provides 3 main chain carbonyls for co-ordination [4]. The gating residue Glu309 provides two to cap the bound Ca^{2+}, and no water molecule contributes to site II. This arrangement of oxygen atoms is reminiscent of the EF-hand motif found in many Ca^{2+}-binding

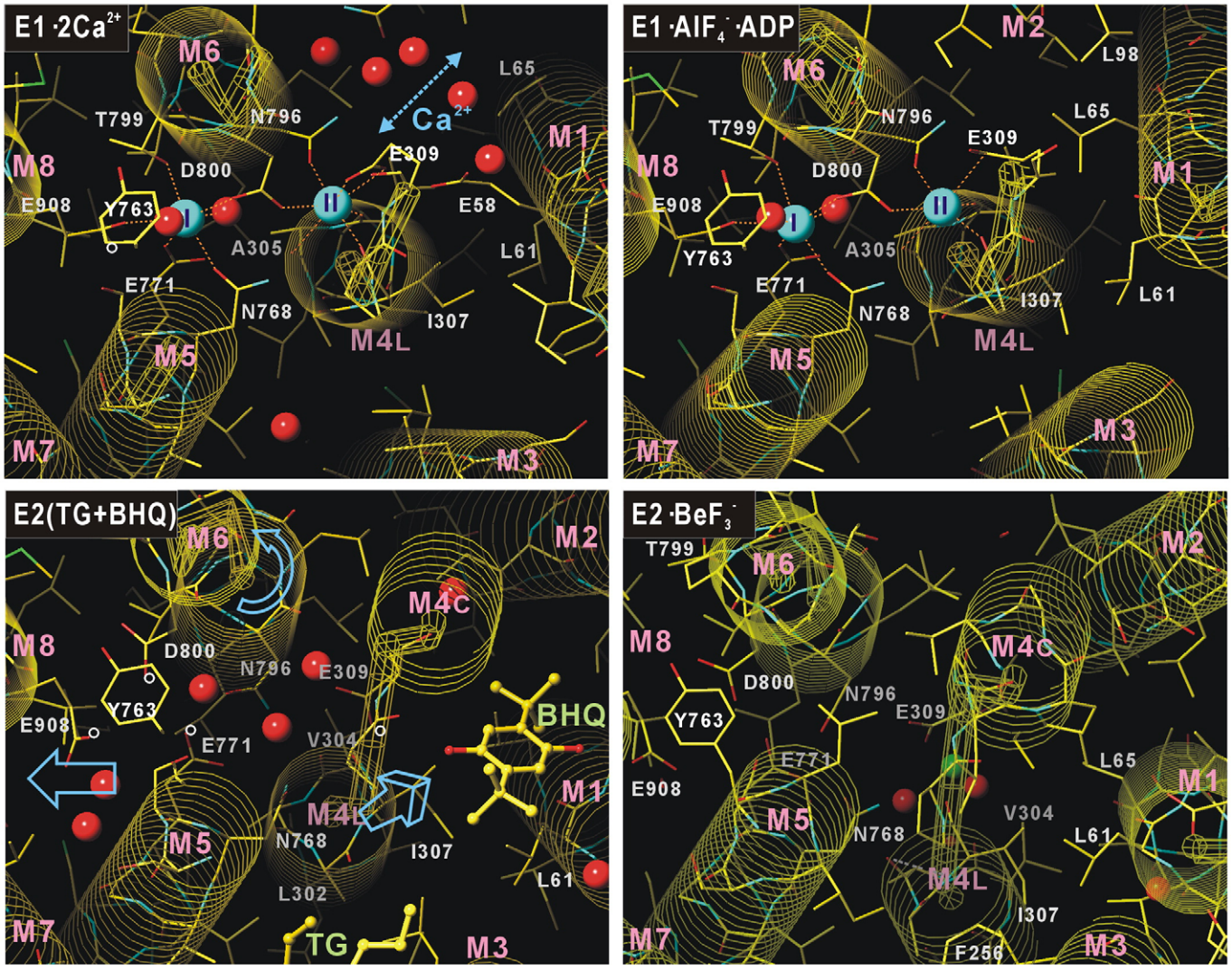

Fig. 2. Details of the transmembrane Ca^{2+}-binding sites in four different states viewed approximately normal to the membrane. Ca^{2+} appear as cyan spheres and water molecules as red spheres. Small white circles in E1·2Ca^{2+} and E2(TG+BHQ) indicate likely protons [8]. Large arrows in E2(TG+BHQ) show the movements of transmembrane helices in the transition from E2(TG+BHQ) to E1·2Ca^{2+}. Two inhibitors, TG (thapsigargin) and BHQ (2,5-di-*tert*-butyl-1,4-dihydroxybenzene), are shown in ball and stick in E2(TG+BHQ). The atomic model for E2·BeF_3^- (analogue of the E2P ground state) is taken from PDB ID: 3B9B [36]; the green sphere is assigned as Mg^{2+}. Note that site II Ca^{2+} is exchangeable by conformation change of the E309 side chain in E1·2Ca^{2+} but not in E1·AlF_4^-·ADP (analogue of E1~P·ADP), as the space around the E309 side chain is occupied by L65 (M1) and other hydrophobic side chains.

proteins (e.g. [29]). Thus, both sites have 7 co-ordinations but of different characteristics.

A key question is how the Ca^{2+}-binding sites and phosphorylation site communicate with each other. In this regard, it is important to note that the cytoplasmic end of M5 is integrated into the P-domain near the phosphorylation site and hydrogen bonded to the M4 helix forming a short β-strand [5]. The P-domain is also connected to M3 with critical hydrogen bonds. Thus, the events that occur at either site can be mechanically transmitted to the other site [5,30].

3. E2 → E1 → E1·2Ca^{2+}: binding of Ca^{2+}

The E2 state, that is, the state subsequent to the release of Ca^{2+} into the lumen and hydrolysis of aspartylphosphate, is considered to be the ground state of the enzyme. The headpiece is compact, as M5 is bowed towards M1 to bring the P-domain underneath the ^{181}TGES loop of the A-domain (Fig. 1b). The P-domain is free from distortion by bound phosphate or Mg^{2+}. The transmembrane Ca^{2+}-binding cavity is filled with water molecules and all the carboxyl groups there are predicted to be protonated to compensate for the space and charge imbalance created by the release of Ca^{2+} [8] (Fig. 2). This structure can persist, however, only at low pH, apparently because the charge compensation by H^+ is insufficient at pH 7. Although the three cytoplasmic domains are associated most closely in this state with several hydrogen bonds between them, thermal agitation opens the headpiece and releases bound protons into the cytoplasm [31]. The released protons are dissipated away through the SR membrane and, at pH 7, placing most of the ATPase molecules into the E1 state [32]. In this state, the carboxyl groups in the Ca^{2+} binding cavity are not protonated and, therefore, have high affinity to Ca^{2+}. In the presence of mM concentration of Mg^{2+} at pH 7, i.e. under the physiological conditions, it is likely that one Mg^{2+} occupies site I when the concentration of cytoplasmic Ca^{2+} is low (note that site I is formed by oxygen atoms of side chains and water, thus likely to be flexible) and evokes a large rearrangement of the transmembrane helices to shorten the time required for forming the E1·2Ca^{2+} state [33]. The structure of the ATPase in the E1·Mg^{2+} state remains to be determined.

Ca^{2+} binding to the ATPase straightens the M5 helix and breaks the closed configuration of the headpiece by bringing the P-domain apart from the A-domain (Fig. 1b). This will allow the delivery of ATP to the phosphorylation site. ATP can bind to the N-domain in the absence of Ca^{2+} and facilitates the opening of the headpiece but

cannot reach Asp351, the phosphorylation residue. Straightening of the M5 helix moves the P-domain and, thereby, the M4 helix towards the cytoplasm by one turn of α-helix [5] so that site II becomes properly formed. Presumably due to deprotonation of Glu771 and Asp800, the unwound part of M6 rotates 90 ° and allows Asp800 to take the proper position (Fig. 2). Two Ca^{2+} enter the high affinity sites through the gating residue Glu309 on M4 in single file. Because there is enough space around Glu309 (Fig. 2), the cytoplasmic gate can still open.

4. E1·2Ca^{2+} → E1P transition: formation of the occluded state

ATP binds near the hinge between the P-and N-domains and crosslinks them, so that the γ-phosphate of ATP and a Mg^{2+} (or Ca^{2+}) bind to the P-domain to bend it in two directions. Extensive hydrogen bonds are formed around ATP and the metal, suggesting that this is a highly strained state. The loop containing Asp703, an absolutely conserved residue in the P-domain, is pulled towards Asp351 by Mg^{2+} binding and bends the P-domain. This bending of the P-domain tilts the A-domain by ~30 °, which sits on the last helix in the P-domain (P7) at one end. The movement of the P7 helix is highly amplified at the opposite end of the A-domain where M3 is connected and places strain on the M3–A-domain link. This strain appears to be the driving force for the A-domain rotation (Fig. 1b), as the proteolysis prevents the E1P → E2P transition [34].

At the same time, the M1 helix is pulled up and bent so that the amphipathic N-terminal part (M1′) lies on the membrane surface. This bending brings the top of the transmembrane part of M1 to occupy the space around Glu309 and, thereby, fixes the conformation of the Glu309 side chain. Thus, the cytoplasmic gate of the Ca^{2+} binding sites is now closed and two Ca^{2+} are occluded in the transmembrane binding sites. The M1 and M2 helices form a V-shaped structure, which moves as a rigid body until the end of the reaction cycle, and transmits the movements of the A-domain to other transmembrane helices, primarily the lumenal half of M4 (M4L, Fig. 1b).

Phosphoryl transfer from the γ-phosphate to Asp351 fixes the N-domain in a highly inclined position so that a mechanical couple is formed between the N- and A-domains in preparation for the next main event, a 90 ° rotation of the A-domain in the E1P–E2P transition. The change in inclination of the N-domain with respect to the P-domain is nearly 90 ° between E1·2Ca^{2+} and E1·AMPPCP crystal structures and apparently exceeds the amount allowed for a hinge bending. A second hinge is integrated into the β-sheet itself [35] and takes care of an extra ~30 ° inclination. The two halves of the β-sheet that constitutes a Rossmann fold of the P-domain are staggered in the absence of phosphate bound to Asp351 but are aligned in E1·ATP through E2·Pi. This movement of the β-sheet is a kind of bending in the direction perpendicular to the plane of the β-sheet, different from the direction of the primary hinge bending movement. Thus the N-domain can form a different interface with the A-domain, or fixes the A-domain in a different orientation from those in the unphosphorylated states.

5. E1P → E2P transition: release of Ca^{2+} into the lumen of SR

Phosphoryl transfer to Asp351 triggers the opening of the N- and P-domain interface [7]. The A-domain rotates 90 ° around an axis ~25 ° inclined from the membrane normal and brings the ^{181}TGES loop of the A-domain deep into the gap between the N- and P-domains above the aspartylphosphate (Fig. 1b) [17,36]. This position of the TGES loop is stabilised by several hydrogen bonds, presumably to make the resident time in this state long enough for releasing the bound Ca^{2+} into the lumen of SR. Mg^{2+} will also contribute to the positioning of the TGES loop through a water molecule. The TGES loop keeps the same E1-type conformation but now occupies the space where ADP was to prevent the binding of ADP [7] and shields the aspartylphosphate from bulk water, as Gly182Cα makes van der Waals contacts with the aspartylphosphate [17].

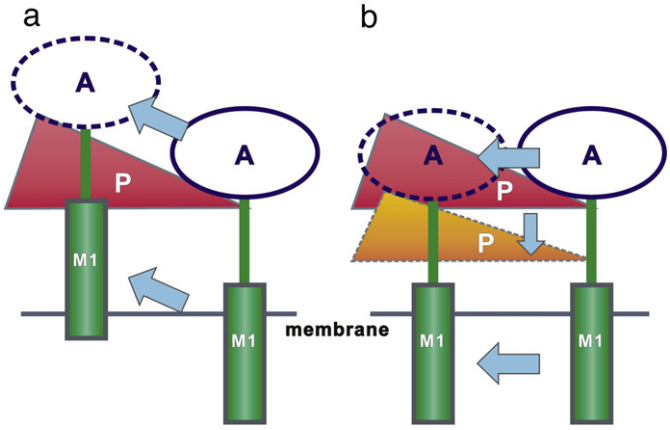

Fig. 3. A cartoon illustrating 2 hypothetical situations for the A-domain rotation. In both cases, the distances between the A-domain and the transmembrane helices (represented by M1) are fixed. In (a), the P-domain is fixed whereas transmembrane helices can come out from the membrane; in (b), the transmembrane helices stay within the membrane whereas the P-domain can move (or incline) in a direction perpendicular to the membrane.

This A-domain rotation causes a drastic rearrangement of the transmembrane helices M1–M6, including a large downward movement of M4, bending of M5 towards M1 (Fig. 1b) and rotation of M6 (Fig. 2), which destroy the Ca^{2+}-binding sites [5,30]. The lower sections of M1 and M2 push against M4L, opening the lumenal gate and releasing the bound Ca^{2+} into the lumen [17]. This will allow protons and water molecules to enter and stabilise the empty Ca^{2+}-binding sites [8].

This rearrangement of transmembrane helices is related to an inclination (~30 °) of the P-domain (Fig. 1b). Because of the wedge shape of the P-domain, when the A-domain rotates, the junctions with the M1 and M2 helices would be raised if the P-domain did not move (Fig. 3a). If that were the case, as the link between the A-domain and M1 has a fixed length, the V-shaped structure formed by M1 and M2 would be further raised from the membrane. Alternatively, the P-domain may incline and keep the junctions at similar heights if the V-shaped structure has to be kept at a similar height with respect to the membrane (Fig. 3b). In reality, M5 can bend at Gly770 and bring the P-domain towards the membrane [5], whereas M1 and the hydrophobic half of M1′ would have a strong tendency to stay in the hydrophobic part of the membrane [19]. As a result, the P-domain inclines and pushes down the M4 helix towards the lumen, just as in the E2(TG) crystal structure [5].

We can now understand why the length of the A-domain–M1′ is critical in processing of aspartylphosphate [19,20]. If it is too short, E1P → E2P transition is blocked. If it is too long, the ATPase cannot release bound Ca^{2+} into the lumen of SR although it can reach E2P (E1P → E2P transition is actually accelerated) and stops there.

6. E2P → E2 transition: hydrolysis of aspartylphosphate and closing of the lumenal gate

In the E2P → E2·Pi transition, the A-domain rotates further by 25 ° around a different axis [17]. During this transition, the lumenal gate becomes closed again [37,38], as the V-shaped structure becomes ~5 Å lower to the lumenal side (Fig. 4) and imposes more upright position on M4L. These movements of the V-shaped structure are caused by disruption of a part of the M2 helix and the change of its path (M2 switch; Fig. 4) [17]. At the position of the A-domain realised in the E2·BeF_3^- (i.e. E2P ground state analogue) crystals, M2 and accordingly the V-shaped structure can take both of these two (i.e. E1 and E2 type)

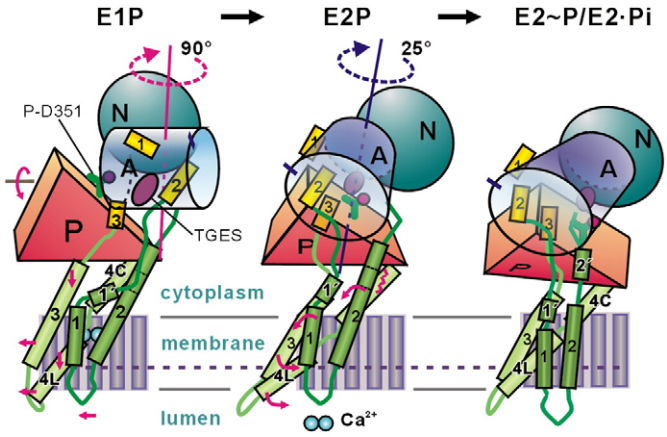

Fig. 4. A cartoon illustrating a 2 step rotation in the processing of aspartylphosphate and lumenal gating of the ion pathway based on 3 crystal structures [17]. Small arrows indicate the movements of the transmembrane helices. M1–M4 (green) and A1–A3 (yellow) helices are numbered. P-D351 refers to phosphorylated Asp351.

conformations. In fact, thapsigargin (TG) can bind to E2·BeF_3^- with little changes in the cytoplasmic domain [17] and close the lumenal gate [37,38]. The lower (E2) position appears energetically more favourable for the V-shaped structure, as the mutant with a longer A–M1′ link preferred the E2 position and formed E2·BeF_3^- keeping Ca^{2+} bound in the transmembrane binding sites [19].

This rotation of the A-domain is related to the introduction of one water molecule in the phosphorylation site [17]. Glu183 now fixes the water molecule and catalyses its attack on the aspartylphosphate [21,22], presumably by withdrawing a hydrogen from the water molecule. The release of the phosphate and Mg^{2+} relaxes the P-domain, and thereby releases the M1 and M2 helices so that M4L closes the lumenal gate completely and places the ATPase into the E2 state.

7. Conclusions

As described, the mechanism of ion pumping by Ca^{2+}-ATPase appears fairly stochastic. In molecular dynamics simulations, we see that the bound Ca^{2+} in E1·2Ca^{2+} undergoes very large thermal fluctuations and continuous water attacks [14]. Glu309 side chain appears to be the only obstacle for a water molecule to replace the Ca^{2+}. Then, it is obvious that the occlusion requires the second seal that fixes the side chain of Glu309. In fact, a helix is employed for this purpose, not just a single residue, to cope with thermal fluctuations. That is the reason why the domain movements are so large and changes in domain interface are used to move the gating machinery (Fig. 1b). ATP, phosphate, Mg^{2+}, Ca^{2+} and even water (and presumably protons also) are used as modifiers of the interfaces.

Energy barriers between the principal intermediates appear to be comparable to the thermal energy, as the key events, e.g., the rotation of the A-domain, occur when ADP is released (or at least detached from the phosphorylation site). We can readily make E1P analogues simply by mixing the ATPase and stable phosphate analogues, such as AlF_4^-, and they even change spontaneously to E2P analogues when the Ca^{2+} concentrations are low [39]. That will be the reason why nearly 100% efficiency of energy utilisation is possible and ATP can be synthesised by backward reaction (e.g. [40]).

We postulated a question on countertransport as "why countertransport of H^+ is necessary despite that the SR membrane is leaky to H^+", and our answer was that "the charge imbalance caused by Ca^{2+} release has to be compensated at least in part by protonation" [8]. In fact, leak of transported H^+ is a part of the reaction cycle. It is necessary to make the Ca^{2+} binding sites into high affinity by thermal agitation. This is an analogous situation to Na^+K^+-ATPase with K^+-leak channels.

It should be also noticed that "placing strain and releasing" is the method used for driving the reaction [18]. For instance, the large rotation of the A-domain is initiated by placing strain on the A-domain–M3 link. This strain is originally placed by Mg^{2+}-binding, which bends the P-domain and thereby changes the orientation of the A-domain to pull the A-domain–M3 link. Because the A-domain–M3 link is flexible, it would be difficult to push the slacked link to put the A-domain into the original position.

Thus, it seems that the overall reaction is made possible by suppressing backward reaction rather than accelerating the forward reaction. In the crystal structures, we have seen such devices integrated into the ATPase [7]. For example, the TGES loop of the A-domain occupies where ADP was in E1·AlF_4^-·ADP to prevent the rebinding. It might also be a part of the reason why co-operative binding is used for Ca^{2+} and for ATP and Mg^{2+}. In the absence of ATP or phosphate (analogue), increasing the concentration of Mg^{2+} to 20 mM does not result in its proper binding to the P-domain; it requires either phosphate or ATP.

We now also understand, to some extent, why the structure of Ca^{2+}-ATPase has to be so. For instance, closed configurations of the cytoplasmic domains are necessary, because Ca^{2+}-ATPase changes domain interfaces to orient the A-domain for regulation of the gating mechanism. In the E2 state, the closed configuration appears to be important in two other aspects: (i) restriction of the delivery of ATP to the phosphorylation site; (ii) restriction of the thermal movements of transmembrane helices [30]. As ATP can bind to the ATPase even in the E2 state and SERCA1 has no mechanism for regulating the reaction cycle other than Ca^{2+} itself, the delivery of ATP γ-phosphate to the phosphorylation site has to be restricted physically. If the A-domain fluctuates too much by thermal energy, Ca^{2+} transferred into the lumen of SR will leak into the cytoplasm. Therefore, the A-domain has to be fixed by other cytoplasmic domains, unless Ca^{2+} itself fixes the transmembrane helices. Even so, such leakage does occur, though small, in the absence of Ca^{2+} and thapsigargin suppresses it. The link between the A-domain and the M1 helix has to be flexible, to provide tolerance to the gating machinery for thermal fluctuation [18]. Yet the lengths of the links are important [20,41], presumably because the size of the A-domain movement is set just enough to surpass thermal fluctuation. Perhaps we are beginning to understand that ion pumps are working in thermally fluctuating world, yet utilising thermal energy effectively for ion transport.

Acknowledgements

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