Comparison of H^+-ATPase and Ca^{2+}-ATPase suggests that a large conformational change initiates P-type ion pump reaction cycles

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Background: Structures have recently been solved at 8 Å resolution for both Ca^{2+}-ATPase from rabbit sarcoplasmic reticulum and H^+-ATPase from *Neurospora crassa*. These cation pumps are two distantly related members of the family of P-type ATPases, which are thought to use similar mechanisms to generate ATP-dependent ion gradients across a variety of cellular membranes. We have undertaken a detailed comparison of the two structures in order to describe their similarities and differences as they bear on their mechanism of active transport.

Results: Our first important finding was that the arrangement of 10 transmembrane helices was remarkably similar in the two molecules. This structural homology strongly supports the notion that these pumps use the same basic mechanism to transport their respective ions. Despite this similarity in the membrane-spanning region, the cytoplasmic regions of the two molecules were very different, both in their disposition relative to the membrane and in the juxtaposition of their various subdomains.

Conclusions: On the basis of the crystallization conditions, we propose that these two crystal structures represent different intermediates in the transport cycle, distinguished by whether cations are bound to their transport sites. Furthermore, we propose that the corresponding conformational change (E_2 to E_1) has two components: the first is an inclination of the main cytoplasmic mass by 20° relative to the membrane-spanning domain; the second is a rearrangement of the domains comprising the cytoplasmic part of the molecules. Accordingly, we present a rough model for this important conformational change, which relays the effects of cation binding within the membrane-spanning domain to the nucleotide-binding site, thus initiating the transport cycle.

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Received: **6 April 1999**
Revised: **24 May 1999**
Accepted: **24 May 1999**

Published: **16 June 1999**

Current Biology 1999, 9:672–679
http://biomednet.com/elecref/0960982200900672

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Background

Members of the P-type family of ion pumps are responsible for maintaining the ionic homeostasis of cells (reviewed by Møller *et al.* [1]). These pumps are thought to use a common mechanism to couple the energy of ATP hydrolysis to ion transport and thus create an electrochemical gradient across the membrane. Many family members have been identified by sequence homology and the most conserved region occurs at the heart of the ATP-binding domain [2,3]. Hydrophobicity plots are also consistent across the family and are suggestive of 10 transmembrane helices. This topology suggests two long loops on the cytoplasmic side of the membrane, one between helices 2 and 3 comprising ~130 residues, and one between helices 4 and 5 of 350–450 residues. This latter stretch alone accounts for ~40% of the polypeptide and contains the catalytic core of the molecule.

Biochemically, P-type ATPases are characterized by the formation of an acid-stable phosphoenzyme as a reaction intermediate during the pumping cycle, and by their inhibition by vanadate. Formation of a high-energy aspartyl phosphate relies on the binding of cytoplasmic ions to the membrane-spanning domain. This energy is then used to drastically lower the affinity for ions and to grant them access to the extracellular side of the membrane; ion release then allows hydrolysis of the low-energy aspartyl phosphate to complete the cycle.

Conformational change is thought to provide the physical coupling between the ion-binding site and phosphorylation site, which are separated by ~40 Å [4,5]. Indeed, it was long ago proposed that P-type ATPases cycle between two main conformations [6–8], designated E_1 and E_2. The E_1 state corresponds to ion sites facing the cytoplasmic side of the membrane and the E_2 state to ion sites facing the extracellular (or lumenal) side. These two conformational states are well characterized in many of the pumps and appear to be related by global changes in tertiary structure involving both membrane-spanning and cytoplasmic parts of the molecule. For instance, the E_1 to E_2 transition induces changes both in intrinsic tryptophan fluorescence [9,10], which predominantly reports the state of the membrane-spanning

domain, and in fluorescence of various probes of the ATP-binding site such as fluorescein isothiocyanate [11]. Further evidence for conformational changes both during the E_2 to E_1 transition (that is, ion binding) and during nucleotide binding and phosphoenzyme formation, comes from low-angle, X-ray scattering, patterns of proteolysis, accessibility to antibody and deuterium exchange [4,12–14].

Structural studies of P-type ATPases should ultimately reveal the nature of these conformational changes. Indeed, recent structures of Ca^{2+}-ATPase [15] and H^+-ATPase [16] at 8 Å resolution revealed 10 transmembrane helices and a strikingly different organization for the cytoplasmic domains. In the present work, we have undertaken a detailed comparison of these two structures. Given the relatively large phylogenetic distance between Ca^{2+}-ATPase and H^+-ATPase, this comparison should identify structural features that are conserved across the whole family of P-type ATPases.

Results

The membrane-spanning domain

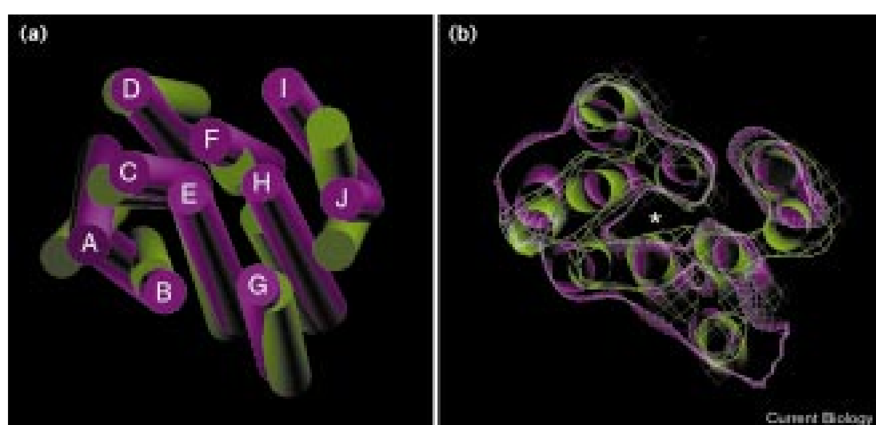
The first indication of structural conservation comes from the independent fitting of both Ca^{2+}-ATPase and H^+-ATPase maps with 10 transmembrane helices, as presented in the original publications [15,16]. Qualitative comparison of the arrangement of these helices indicated similar features, in particular a distinctive right-handed bundle of four helices (A, B, C and E in the original nomenclature of the H^+-ATPase, which will be used throughout this paper), lined by a row of four helices (D, F, G and H) on one side and another V-shaped pair of helices (I and J) further out. To carry out a more detailed comparison, we used the graphics program O [17] to move Ca^{2+}-ATPase helices as a rigid group of straight cylinders to match the H^+-ATPase map.

The Ca^{2+}-ATPase helices fitted the H^+-ATPase map rather well, and it was therefore clear that the two molecules had a very similar structure within the membrane (Figure 1). Relatively minor differences can be explained by slightly different inclination angles for the individual helices, many of which are probably because of ambiguities in fitting helices to an 8 Å map. In general, this fit was less ambiguous for H^+-ATPase because of the better signal-to-noise ratio at high resolution that more clearly resolved individual helices in cross-sections through the membrane-spanning domain. Nevertheless, a second, independent map of Ca^{2+}-ATPase at 8 Å resolution (H.S. Young and D.L.S., unpublished observations) indicates that all the important details of the original Ca^{2+}-ATPase map are reproducible. For both H^+-ATPase and Ca^{2+}-ATPase, certain helices are defined better than the others and this trend seems consistent in both maps: for example, helices E and G are particularly well defined, whereas A and H are less well defined owing to broad and weak densities, respectively. A V-shaped density for helices I and J is present in both maps but the individual helices have somewhat different angles, producing a poor match near the cytoplasmic surface. Furthermore, a region of low density was seen within the membrane-spanning domain of Ca^{2+}-ATPase and was proposed to provide access for ions from the lumenal side of the membrane to a site in the center of the four-helix bundle [15]. At a density threshold similar to that of the Ca^{2+}-ATPase, the map of the H^+-ATPase also reveals a small cavity in the same location (asterisk in Figure 1).

Cytoplasmic domains

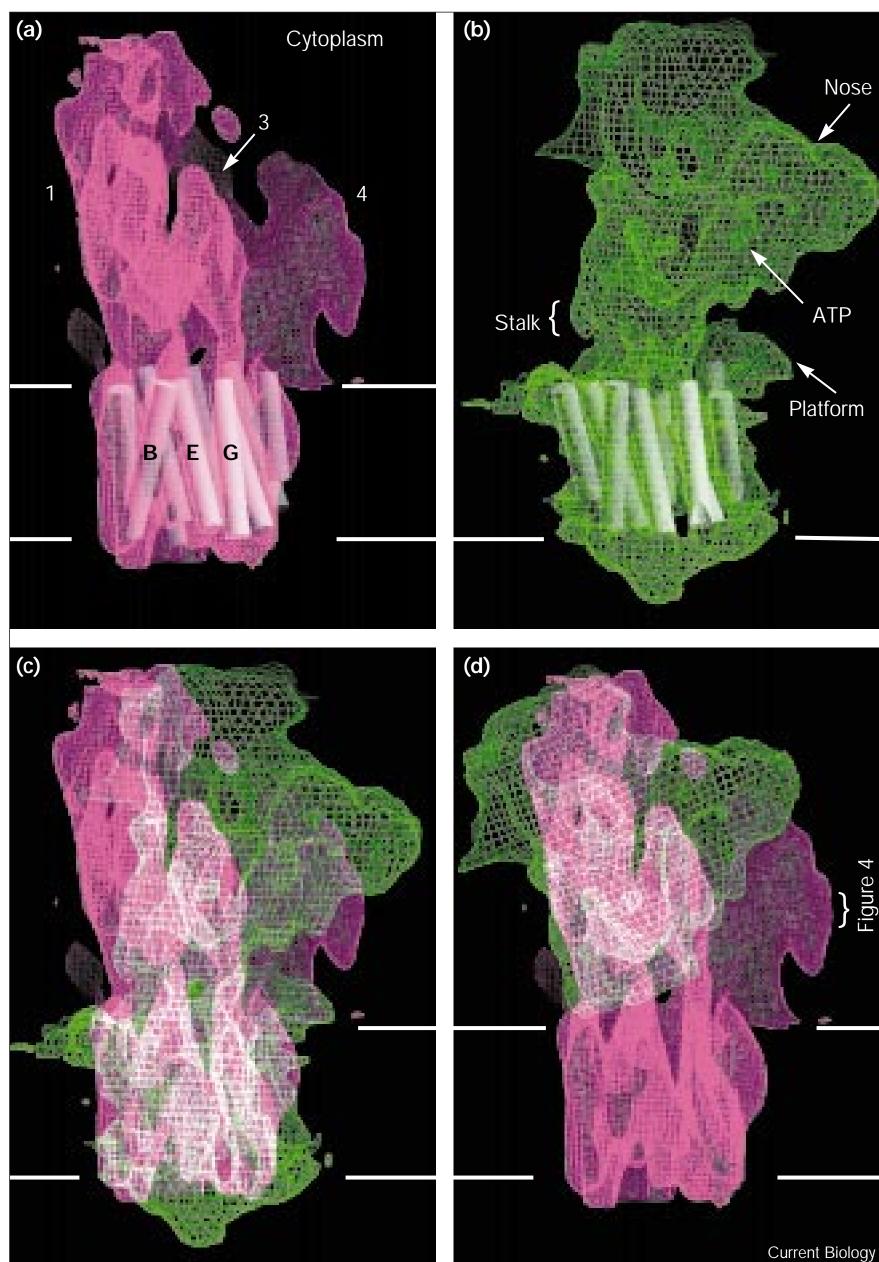
The marked similarity in the arrangement of transmembrane helices led us to a more thorough comparison of the cytoplasmic domains. In both molecules, the overall

Figure 1



Overlay of the membrane-spanning domains of H^+-ATPase (purple) and Ca^{2+}-ATPase (green). **(a)** Cylinders correspond to α helices that had been independently fitted to each map. The alignment was done manually as described in the text; a more quantitative, least-squares alignment of the α carbons to define the two sets of helices was not possible because, at 8 Å resolution, α carbon positions are not defined. The letters correspond to the nomenclature of Auer and colleagues [16] and the view is from the cytoplasmic side of the membrane. **(b)** An 8 Å section from the middle of the membrane showing molecular envelopes together with the fitted helices. The asterisk indicates the channel observed in the Ca^{2+}-ATPase structure leading from the lumenal side of the membrane to a site between helices B, C and E [15].

Figure 2

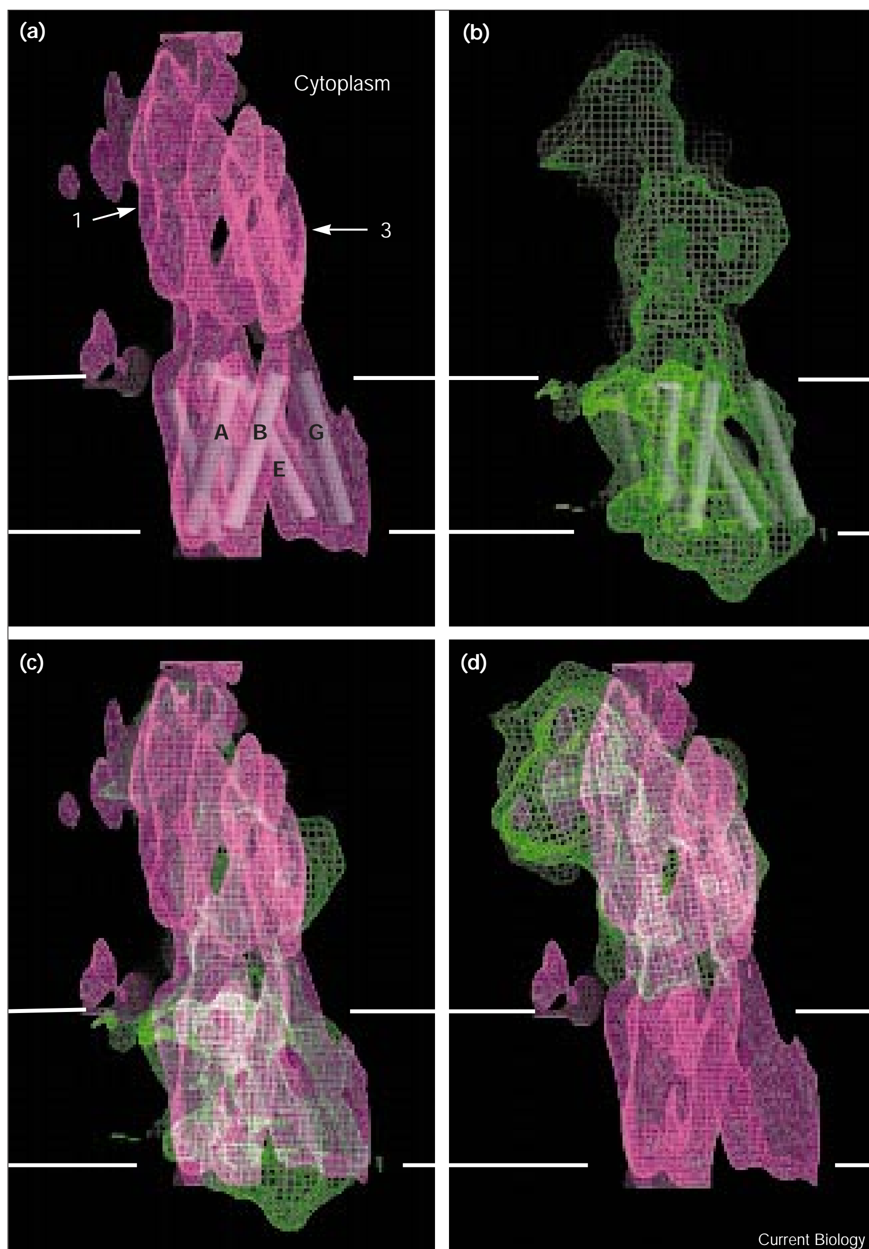


Comparison of the molecular envelopes. **(a,b)** Envelopes for (a) H^+-ATPase and (b) Ca^{2+}-ATPase, respectively, with fitted helices shown as cylinders; horizontal white lines delineate the membrane. The various domains are indicated for H^+-ATPase; these were labelled 1–4, although domain 2 is not shown. For Ca^{2+}-ATPase, the stalk, nose, platform and ATP-binding site have all been used as landmarks. **(c)** Overlay of the two molecular envelopes after aligning the membrane-spanning domains; the white net indicates the overlap of pink and green nets. An inclination of the cytoplasmic head of Ca^{2+}-ATPase relative to H^+-ATPase is evident. **(d)** Alignment of the cytoplasmic head of Ca^{2+}-ATPase independent of the membrane-spanning domain, which primarily involved a 20° inclination of the head. The bracket indicates the level of the section shown in Figure 4.

sizes of these domains are consistent with predictions from sequence analyses (~65% of the molecular mass). Both structures have a strong column of density extending above transmembrane helices B–F, but these columns rise from the membrane surface at a different angle in the two structures (Figure 2). This column is likely to comprise the stalk as well as the highly conserved phosphorylation and hinge domains delineated by sequence analysis [18].

For a better match of this central column, the cytoplasmic head of Ca^{2+}-ATPase was severed from the membrane-spanning domain and aligned manually with the H^+-ATPase map. As suggested previously [19], we were able to achieve a good fit for the molecular envelopes by inclining the Ca^{2+}-ATPase head by 20° as shown in Figure 2d. In particular, the column of density comprising domain 1 of H^+-ATPase [16] is well aligned from the membrane surface almost up to the top of the molecule and domain 3 is aligned with a strong protrusion from Ca^{2+}-ATPase (Figures 3,4). In contrast, domains 2 and 4 of H^+-ATPase as well as the cytoplasmic nose and another cytoplasmic domain near the top of Ca^{2+}-ATPase (Figure 2b) were unmatched (Figure 4).

Figure 3



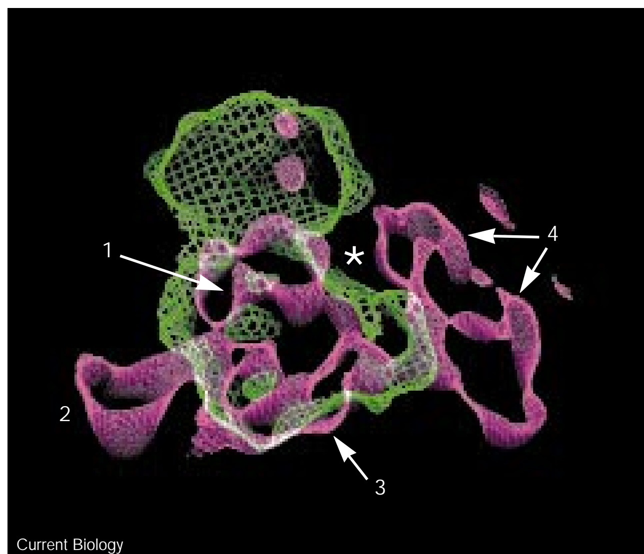
Comparison of the molecular envelopes. The molecules in Figure 2 have been rotated by 90° (that is, molecules in Figure 2 are viewed from the left). **(a)** H^+-ATPase; **(b)** Ca^{2+}-ATPase; **(c)** overlay of molecular envelopes for H^+-ATPase and Ca^{2+}-ATPase; **(d)** alignment of the cytoplasmic head of Ca^{2+}-ATPase independent of the membrane-spanning domain.

The hinge for tilting the cytoplasmic head appears to be at the interface between the stalk and the transmembrane helices and, as a result, the two maps are somewhat different at this interface. In Ca^{2+}-ATPase, the connection between transmembrane helices and the cytoplasmic domains consists of four distinct densities that are more-or-less continuous with the cytoplasmic ends of transmembrane helices B, E, F and G [15]. We presume these densities to be helices and refer to them as stalk helices 2 to 5 (S2–S5). In H^+-ATPase, helices E and G are clearly connected to the cytoplasmic part of the molecule in a similar way. A more tenuous connection can also be postulated for helix F and possibly for helix B (Figure 3a). Helix C of H^+-ATPase might continue into the cytoplasmic part, however, which would represent an apparent difference from Ca^{2+}-ATPase.

Discussion

By aligning the membrane-spanning domains of H^+-ATPase and Ca^{2+}-ATPase, we have shown that the arrangement of transmembrane helices is surprisingly similar for these two distantly related P-type ATPases. In contrast, the organization of the cytoplasmic domains is strikingly different, making it important to consider the basis for similarities

Figure 4



Overlay of the core domains. This is an 8 Å thick section parallel to and ~20 Å above the cytoplasmic membrane surface (level shown in Figure 2d). The domains of H^+-ATPase are indicated and we propose that the catalytic core consists of domains 1 and 3, which match the Ca^{2+}-ATPase density well. The site of the chromium (Cr)-ATP difference density for Ca^{2+}-ATPase (asterisk between domains 1 and 4 [5]) is consistent with this proposal. Domain 2 of H^+-ATPase and the top-most domain of Ca^{2+}-ATPase do not match anything after alignment. As indicated in Figure 5, we propose a conformational change that would suggest equivalence of domain 4 of H^+-ATPase with the nose of Ca^{2+}-ATPase.

and differences. Overall, the sequences of the two molecules are 27% identical and the strongest homologies occur in the places that define the family of P-type ATPases [2,20]: the site of phosphorylation just following stalk helix S4 (CSDKTGTLT, single-letter amino-acid notation), the ‘hinge’ domain just before S5 (AMTGDGVNDAPxLKKA), as well as shorter sequences in the nucleotide-binding domain (that is, DPPR, MxTGD) and in sequences between S2 and S3 (VVPGDI and VDQSxLTGES). The hydrophobicity profile is also similar, and both amino and carboxyl termini are cytoplasmic, suggesting that the transmembrane topology is similar [1,2].

Major differences include an extra 65 residues on the amino terminus of H^+-ATPase that have a high concentration of negative charges, and an extra 30 residues on the carboxyl terminus of H^+-ATPase; Ca^{2+}-ATPase also has several inserts in the main cytoplasmic loop. Sequence similarities in the membrane-spanning domain are rather limited and only one out of five residues critical for calcium occlusion in Ca^{2+}-ATPase [21,22] are conserved in H^+-ATPase. Nevertheless, a stretch of transmembrane helix 4, which contributes to the calcium-binding site, is well conserved and mutations along this stretch significantly reduce H^+ transport by H^+-ATPase [23]. Also, these pumps share the phenomenology of orthovanadate inhibition and well defined E_1 and E_2 conformations with other members of the family, leading to a broad consensus that the pumps use a similar reaction sequence and similar mechanisms of energy transduction [1,24,25]. Indeed, the striking structural similarity we have observed in the arrangement of helices in the membrane-spanning domains very much supports this consensus.

If this is so, then the gross differences between the cytoplasmic structures might be explained in terms of the conformational changes that drive the reaction cycle. First, it is necessary to consider which of the several different conformational states are present in the crystals used for structure determination. In the case of Ca^{2+}-ATPase, a large number of studies have addressed this question. Vanadate was initially used for crystallization because of its documented ability to lock the enzyme in the phosphoenzyme E_2-P conformation [26]. It was subsequently shown, however, that decavanadate was the relevant species for promoting crystallization and could do so in the absence of orthovanadate [27]. Later, after being characterized as a dead-end inhibitor of the E_2 conformation, thapsigargin was shown to strongly promote the formation of tubular Ca^{2+}-ATPase crystals [28]. This observation, together with the requirement for very low calcium concentrations, suggested that crystallized Ca^{2+}-ATPase was in the E_2 conformation. This conclusion was further tested by buffering calcium concentrations during crystallization, which demonstrated that the calcium sensitivity of crystallization was identical to the calcium requirement for converting E_2 to E_1 (pCa 6.5 [29]). Once calcium sites become saturated, another crystal form with completely different intermolecular contacts is generated, presumably from the E_1 conformation [29–32]. In the case of H^+-ATPase, crystals were grown at pH 6.8 in the absence of any ligands at the nucleotide-binding site [33]. Although H^+-ATPase has a well-characterized pH dependence with maximal activity at pH 6 [34], measurement of a pK_a for transported protons is complicated by a pH effect on ATP affinity and, at more extreme pH, on enzyme stability. Nevertheless, this pK_a is likely to be ~7 [10,35] and the transport sites are therefore likely to be occupied by protons under the conditions of H^+-ATPase crystallization. This situation would produce the E_1 state, which is in fact consistent with studies of proteolytic cleavage under similar conditions [36] as well as with the various effects of ATP-site ligands [14].

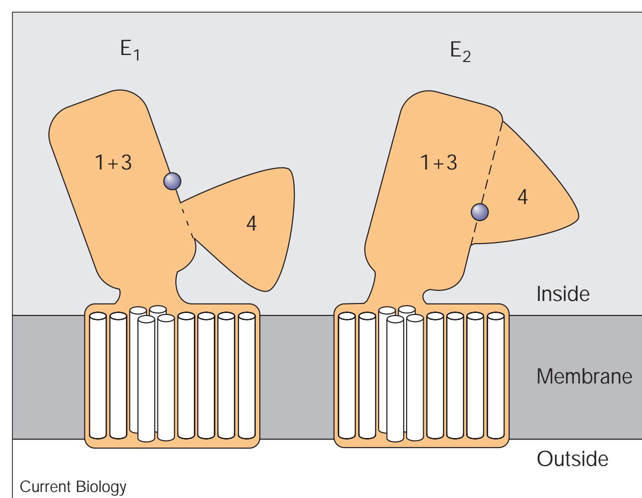
These considerations suggest that at least some of the differences between the cytoplasmic structures are attributable to the E_2 to E_1 transition. In fact, similar changes were suggested by comparing an edge-on projection map of Ca^{2+}-ATPase in the presence of calcium (that is, in the E_1 conformation) [37,38] with the 14 Å map of Ca^{2+}-ATPase in the E_2 conformation [39]. In particular, the edge-on projection suggests that the main cytoplasmic portion inclines

relative to the membrane-spanning domain and that a cleft develops between this density and the cytoplasmic nose; this nose is ~40 Å above the membrane in the three-dimensional map (Figure 2b), but appears to move down closer to the membrane in the projection map, taking up a position similar to that of domain 4 of H^+-ATPase. Thus, this projection map of Ca^{2+}-ATPase in the E_1 conformation bears a striking resemblance to the structure of H^+-ATPase.

The transition from the E_2 to the E_1 conformation would thus have two main components: rearrangement of the cytoplasmic domains and tilting of the whole cytoplasmic head relative to the membrane-spanning domain, as suggested in the schematic drawing in Figure 5. The rearrangement of the cytoplasmic domains would convert the closed, wedge-shaped structure seen in the E_2 state of Ca^{2+}-ATPase to the open arrangement of the domains visible in the cytoplasmic portion of the H^+-ATPase and suggested by the edge-on projection of Ca^{2+}-ATPase in the presence of calcium. In the course of this rearrangement, domain 4 breaks away from the central column of domains 1 and 3 and moves down towards the membrane, where it seems to contact the ‘platform’ that resides just above the membrane surface in the Ca^{2+}-ATPase map (Figure 2b). Comparing the two maps, it is easy to see how domains 1, 3 and 4 of H^+-ATPase would combine into the compact volume of the Ca^{2+}-ATPase head. Domain 2 of the H^+-ATPase seems to be mainly responsible for the formation of stable hexamers, which are characteristic of this enzyme, by forming a link between adjacent cytoplasmic regions. This might explain its apparent absence in Ca^{2+}-ATPase.

Domains 1 and 3 seem to be rather rigid and do not undergo any significant rearrangement, making them good candidates for the catalytic core of the molecule. A recent sequence comparison [40] between P-type ATPases and a superfamily of hydrolases suggest that they have a common fold for their catalytic core domains. Furthermore, family members all utilize a transient covalent bond between the ligand and a conserved aspartate in the catalytic loop [41], supporting the notion of a conserved fold. On the basis of the X-ray structures of two L-2 haloacid dehalogenases [42,43], this catalytic domain has a Rossmann fold with a large insert after the first β-strand. On the basis of the sequence homology, the amino and carboxyl termini of the Rossmann fold are closely linked to the S4 and S5 stalk helices, placing this domain directly above the four-helix stalk seen in the Ca^{2+}-ATPase map in the region of greatest similarity to H^+-ATPase (Figure 4). The best fit of the dehalogenase structure to the cytoplasmic core of Ca^{2+}-ATPase puts the catalytic aspartate near the nucleotide-binding site, which was previously identified in difference maps produced by Cr (chromium)-ATP [5]. The corresponding site in H^+-ATPase is the crevice between domains 1 and 4 (asterisk in Figure 4), suggesting that this crevice represents the ATP site in the E_1 conformation. A more detailed analysis of the structural homology between these dehalogenases and Ca^{2+}-ATPase is currently underway (D.L.S. and N.M. Green, unpublished observations).

Figure 5



A schematic diagram of the conformational change relating the cytoplasmic domains of Ca^{2+}-ATPase and H^+-ATPase, which we propose corresponds to the E_2 to E_1 transition. This involves inclination of the cytoplasmic head by 20° and swiveling of the cytoplasmic nose down towards the membrane surface. The sphere at the domain interface represents the putative ATP-binding site.

Remarkably, the overall arrangement of the 10 transmembrane helices remains unaffected by these major structural changes within the cytoplasmic domains. Apparent differences between less defined helices (A, D, F, I and J) might be because of the uncertainty of fitting α helices to 8 Å density maps, but it is probable that real differences are reflected in the different orientations of the well-defined helices (B and C). Because even these differences are small, and because the current structures come from phylogenetically distant molecules with different ion specificities, we must wait until high-resolution structures are available for at least one of the two molecules in both conformational states before drawing detailed mechanistic conclusions. It is worth noting, however, that the structural changes required for ion pumping need not be large. The recent analysis of bacteriorhodopsin, a light-driven proton pump, at various stages of its proton-pumping cycle has revealed that a displacement of a single transmembrane helix by 2–3 Å on one membrane surface, equivalent to a tilt of no more than 3–5°, is sufficient to elicit proton pumping by altering accessibility and affinity for protons at their binding site (S. Subramaniam and R. Henderson, personal communication). Cation pumping by a P-type ATPase might be accomplished by a similarly small rearrangement of its transmembrane helices.

Unlike the chromophore in bacteriorhodopsin, however, P-type ATPases harness the energy for pumping at a large distance from the cation-binding sites. This requires structural elements to couple the two sites, which must involve the connection between the cytoplasmic and ATP-binding domains and the transmembrane helices involved in ion transport. The large domain movements that we propose to result from ion binding might be analogous to the long-range allosteric changes generated by nucleotide binding and phosphorylation in other well-known biological systems such as glycogen phosphorylase [44] and myosin [45]. Myosin in particular undergoes a massive conformational change, which is amplified by a long lever arm and ultimately results in the power stroke of muscle contraction. Such examples offer a precedent for large structural changes as a way of coupling two activities at a relatively long distance across a protein molecule.

Conclusions

We have shown a marked similarity in the arrangement of the 10 transmembrane helices that compose the membrane-spanning regions of Ca^{2+}-ATPase and H^+-ATPase, despite their low sequence homology. This similarity supports the notion of a common architecture and mechanism for members of the P-type ion-transporting ATPases. The sequence homology that defines the family exists within the large cytoplasmic loops, but the corresponding structures in Ca^{2+}-ATPase and H^+-ATPase appear to be rather different. On the basis of the conditions of crystallization, we conclude that the two structures most probably represent two distinct conformational states, distinguished by whether cations are bound at their transport sites. Thus, the structural differences we have observed in the cytoplasmic regions might reflect the conformational dynamics that functionally link the transport sites in the membrane with the site of ATP hydrolysis in the cytoplasmic region. In particular, the main structural differences consist of a 20° inclination of the main cytoplasmic domain relative to the membrane-spanning domain, and of a rearrangement of the cytoplasmic domain composing the nose of the molecule. According to our hypothesis, this conformational change alters the structure of the ATP-binding site in response to ion binding, thus initiating the reaction cycle that ultimately leads to active transport of the ion across the membrane.

Acknowledgements

This work was supported by NIH grants AR40997 and GM56960 to D.L.S. and by a grant from the DFG to W.K..

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