P-type ion pumps: structure determination may soon catch up with structure predictions

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Three new crystal forms of three different P-type ion pumps offer prospects for higher-resolution structure, especially if the technique of frozen-hydrated electron microscopy is used. Meanwhile, various indirect methods are being used to determine the number of transmembrane helices and their positions in the amino acid sequence.

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Introduction

ATP-driven ion pumps fall into three families, which are known as F-type (named after F1/F0 ATPase), V-type (named after the vacuolar H+ pump), and P-type (those forming a covalent phosphoenzyme). This review focuses on P-type ion pumps, a family that shares both amino acid homologue and a basic reaction mechanism. The family includes a wide range of species (including bacteria, yeast and mammals) and displays an extraordinary diversity in ion specificity and stoichiometry. The best known members of the family are Ca2+-ATPase from the sarcoplasmic reticulum and Na+/K+-ATPase from plasma membranes, both of which have a long history of investigation. Besides detailed studies of the reaction cycle, many techniques have been used to link features of this cycle to molecular structure, e.g. effects of site-specific labels, fluorescence of labels and of intrinsic tryptophan residues, infrared spectroscopy, proteolysis, crosslinking and antibody binding. An important template for considering the resultant flood of biophysical data has been obtained more recently from the cloning and deduction of amino acid sequences for several P-type pumps. These sequences have been used to predict secondary structure and, when considered together with various biophysical data, have led to a detailed model for a consensus structure of the molecule (Fig. 1). The roles of individual amino acids have recently been investigated in studies on a large number of site-directed mutations of Ca2+-ATPase and their effects on fundamental assays such as Ca2+ transport and phosphorylation. Thus, phosphorylation and ATP-binding sites have been confirmed, several residues in the center of the transmembrane domain have been implicated in Ca2+ binding and residues involved in the transduction of energy between these sites have been identified.

Direct structural studies: electron microscopy and X-ray diffraction

Better crystals and better techniques for electron microscopy

Several three-dimensional reconstructions, both of Ca2+-ATPase, and of Na+/K+-ATPase, have been presented over the last seven years. These reconstructions, which have come from two-dimensional crystals, have established the general shape of the cytoplasmic portion of the molecule, but have revealed neither the secondary structure nor a reliable shape for the intramembranous domain. These limitations result from both the use of a negative stain and to the relatively poor order and limited size of the crystals. However, new crystal forms have been recently discovered for Ca2+-ATPase, Na+/K+-ATPase and the gastric H+/K+-ATPase which, combined with the use of frozen-hydrated specimens, provide hope for more informative reconstructions.

Small, three-dimensional crystals of Ca2+-ATPase were recently grown by Dux et al. [1] after solubilizing Ca2+-ATPase in detergent (C12E8). These crystals consist of a stack of two-dimensional crystals, and after altering the preparation somewhat, larger crystals (1–3 μm in diameter) were obtained which were only 3–10 layers thick (500–1600 Å); thus, the thinnest of these crystals (three to five layers) are well suited to electron microscopy. With negatively stained specimens, the symmetry of the crystals (space group C2) and the arrangement of molecules within the crystals was determined [2*]. Next, the crystals were rapidly frozen, thereby preserving native structure in a layer of amorphous ice. Electron microscopy of the crystals in this frozen state (dubbed frozen-hydrated) proved enormously successful and electron diffraction was obtained at 4 Å resolution and images at 6 Å resolution in projection [3**]. The resulting projection map...
was compared to that obtained from negatively stained specimens, and an area with extra density was specifically attributed to the intramembranous domain (as it was assumed that negative stain did not penetrate the membrane). On the basis of this assignment and previous predictions of ten transmembrane helices [4], two rows of five helices each were proposed for the transmembrane domain. Currently, efforts are underway to collect three-dimensional information from these crystals in order to obtain direct evidence on the number and the arrangement not only of these transmembrane helices, but also of the subdomains that compose the cytoplasmic portion.
A new two-dimensional crystal of Na\(^+/K\)+-ATPase was discovered by Skriver et al. [5] after incubating isolated membranes with Co(NH\(_3\))\(_4\)ATP (a stable Mg-ATP analogue). The fact that Co(NH\(_3\))\(_4\)ATP locks the molecule in the E\(_2\)-ATP conformation appears to induce new and apparently more stable crystal contacts. As with previously studied crystals of Na\(^+/K\)+-ATPase, a given sample is heterogeneous, containing non-crystalline membranes; crystalline membranes with p21 symmetry and variable unit cell dimensions; and crystalline membranes with p\(_4\) symmetry. Previous studies used a different p21 crystal form (induced either by vanadate/EGTA/Mg\(_2^+\) or by phospholipase) and suffered from the variability in their cell dimensions [6]; therefore, the invariant cell dimensions of the p\(_4\) crystals provide a better specimen for three-dimensional reconstruction. In fact this reconstruction is underway and the preliminary results with negatively stained specimens have been presented in a recent abstract [7].

New crystals of the gastric H\(^+/K\)+-ATPase have been reported in an abstract by Mohraz et al. [8] and are different from the p21 crystals induced by vanadate, Mg\(_2^+\) and dimethylsulphoxide [9]. As in their work on Na\(^+/K\)+-ATPase [10], Mohraz et al. digested native membranes with phospholipase and in this case produced large (2 \(\mu\)m in diameter) well ordered sheets (20 \(\AA\) resolution in negative stain) with a reported symmetry of p22\(_2\). This symmetry indicates that unlike two-dimensional crystals of Na\(^+/K\)+-ATPase and Ca\(^{2+}\)-ATPase, molecules protrude from both surfaces of the bilayer. The crystals have a tendency to stack, but the authors have identified a 'basic sheet', and three-dimensional reconstruction of negatively stained specimens is underway [11]. Because these crystals are so large and apparently well ordered, it would be practicable to study them in the frozen-hydrated state.

Finally, a new reconstruction of the well studied p21 crystals of Ca\(^{2+}\)-ATPase (induced by vanadate, Mg\(_2^+\) and EGTA) is being performed with frozen-hydrated specimens (DL Stokes and C Toyoshima, unpublished data). In solution, the constraints of the lattice on the surface of a vesicle cause a thin tube to form, which has a helical arrangement of Ca\(^{2+}\)-ATPase; only after the collapse of these tubes (i.e. after staining and drying) can the crystals be treated as two-dimensional, as Taylor et al. [12] have done in their reconstruction. Castellani et al. [13] did perform a helical reconstruction on tubes that had been filled with negative stain, but rapid freezing is a much better way of preserving the helical symmetry, yielding 14 \(\AA\) resolution in our reconstructions of frozen-hydrated tubes versus 30 \(\AA\) for stained tubes. The advantage of helical reconstruction over the move common tilt construction is that all Fourier data is present in a single helical image; even after lengthy data collection of many tils from a two-dimensional crystal, a 60\(^\circ\) cone of data is inevitably missing, which blurs the reconstruction normal to the plane of the membrane [14]. Thus, in reconstructions from each individual helical tube, the boundary of the lipid bilayer and the shape of protein domains on either side are easily visible. After averaging together several such reconstructions, we also expect to see the shape of the intramembranous protein domain, though the resolution is not high enough to resolve individual helices. When completed, this should give the most detailed structure of the Ca\(^{2+}\)-ATPase obtained so far, with clearly delineated domains both inside and outside the lipid bilayer.

Effect of conformational state on crystallization

At least three members of the P-type ATPase family form two-dimensional crystals with p21 symmetry under conditions that stabilize the E\(_2\) conformation (vanadate, Mg\(_2^+\) and either EGTA or dimethylsulphoxide). Therefore, this crystal form is likely to consist of molecules that are locked into the E\(_2\) (or perhaps E\(_2\)-P) conformation. By similar reasoning, crystals of Ca\(^{2+}\)-ATPase induced either by Ca\(^{2+}\) (10 \(\mu\)M) or by lanthanides (a few \(\mu\)M) appear to be made up of molecules in the E\(_2\)-ATP conformation and the different crystal symmetry (p1, resulting from different molecular contacts) is thought to reflect this difference in molecular conformation [15]. In addition, the E\(_2\)-ATP conformation of Na\(^+/K\)+-ATPase is stabilized by Co(NH\(_3\))\(_4\)ATP, which also induces a different set of crystalline contacts [5]. These different crystal forms make it possible to compare the structure of the molecules in different conformations, and it is therefore important to verify precisely which reaction intermediate is present in a given crystal form.

Recently, two papers have addressed crystallization patterns of various reaction intermediates in scallop sarcoplasmic reticulum, which contains p21 crystals in situ as well as in vitro in the absence of any special inhibitors (i.e. in relaxing conditions [16]). Because the crystal packing is identical to that of vanadate-induced p21 crystals from rabbit sarcoplasmic reticulum, it seems likely that the molecular conformation of these two closely related Ca\(^{2+}\)-ATPase molecules is the same. Hardwicke and Bozzola [17] studied the effect of phosphorylation on this p21 lattice, and determined that phosphorylation with either inorganic phosphate (yielding E\(_2\)-P) or ATP (yielding E\(_1\)-P) produced a different crystal lattice with p1 symmetry. Addition of either \(\beta\gamma\)-methylene adenosine 5'-triphosphate (AMP-PCP) or 5'-adenylylimidodiphosphate (AMP-PNP) did not disrupt the p21 lattice. Castellani et al. [16] studied the effect of Ca\(^{2+}\) and observed that saturating levels (pCa > 5.5) had no effect on the p21 lattice. Higher concentrations of Ca\(^{2+}\) did disrupt the lattice, but because the high-affinity Ca\(^{2+}\) sites had already been saturated, this disruption was most likely due to secondary effects and not a conformational change induced by Ca\(^{2+}\) binding. Thus, for scallop sarcoplasmic reticulum, it seems that E\(_1\)-Ca\(_2\), E\(_2\) and E\(_2\)-ATP all result in p21 crystals, and that E\(_1\)-P and E\(_2\)-P result in p1 crystals. No image analysis was performed on the p1 crystals, so it is not clear whether or
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not they represent the same p1 crystal form, that can be induced in rabbit sarcoplasmic reticulum by lanthanides or Ca$^{2+}$ [18]. In relating this to rabbit sarcoplasmic reticulum, there are several points to consider: an oligomeric form of vanadate (decavanadate) is responsible for crystallization but monomeric vanadate inhibits and stabilizes E$_2$ [19]; p21 crystals of Na$^+$/K$^+$-ATPase can be induced by phospholipase in the absence of vanadate [10]; and finally, decavanadate-induced crystallization of Ca$^{2+}$-ATPase is successful even when Cr-ATP is occupying the active site (J.J. Lacapere and DL Stokes, unpublished data).

Thus, the previous correlations between crystallization conditions and molecular conformation within crystals may have been too simplistic.

To make matters worse, a careful analysis of p21 arrays of Na$^+$/K$^+$-ATPase indicates that this lattice may contain two distinct conformations. Beall et al. [20] performed real-space correlation averaging, after which the two 'symmetry-related' molecules appeared significantly different in projection. Hebert et al. [21] verified this breakdown in p21 symmetry and presented a three-dimensional reconstruction from a single crystal, indicating that the twofold symmetry is reasonably good at the centre of the reconstruction (i.e. near the membrane), but breaks down as one moves away from this centre (i.e. out into the cytoplasm and into the extracellular medium). Beall et al. [20] point out that this structural asymmetry may be correlated with proteolysis experiments by Mohraz et al. [22], which suggest the presence of two different molecular conformations coexisting in the crystals; alternatively, Beall et al. [20] cite the presence of two species of the α-subunit with a slightly different p1 as a basis for the asymmetry [23]. As the asymmetry is also visible in frozen-hydrated specimens [24], it cannot be an artefact of staining. It is unclear why the asymmetry was not seen by Mohraz et al. [25] in their careful three-dimensional reconstruction; they claimed to have observed good twofold symmetry in all their images and their reported power loss of <1% upon symmetrization supports this claim.

**X-ray diffraction of sarcoplasmic reticulum**

Blasie and co-workers have added two reports to their series of X-ray diffraction experiments on orientated pellets of sarcoplasmic reticulum. The first report addresses structural changes in the mass distribution across the membrane upon phosphorylation [26]. Their original experiment [27] showed a redistribution of 8% of the mass from the cytoplasmic head into the hydrophobic core; subsequently, they investigated low temperature [28] and low concentrations of Mg$^{2+}$ [29] as ways to slow down the reaction cycle, thereby providing more time to record the X-ray diffraction pattern from E$_1$ ~ P. However, both of these conditions caused redistributions of mass of the same magnitude as those described for phosphorylation. In the latest paper, Blasie et al. [20] showed that the mass redistribution resulting from phosphorylation occurs in the opposite direction to those caused by low temperatures and low Mg$^{2+}$ concentration. The second paper describes how the location of the high-affinity Ca$^{2+}$-binding sites was identified by substituting lanthanide ions (24 μM La$^{3+}$ or Tb$^{3+}$) for Ca$^{2+}$ and recording X-ray diffraction near the absorption edge for each ion at a resolution of 40 Å [30]. After modelling the location of the ions in the electron-density profile as three discrete points (5 Å wide), they found that 80% of the electron density occurred at a position 12 Å above the cytoplasmic surface of the membrane, which is at the top of the thin 'stalk' that connects the massive cytoplasmic head to the membrane. About 20% of the ions are seen on either surface of the membrane, quite possibly interacting with phosphatidylserine. The authors [30] associate the major peak with a high-affinity Ca$^{2+}$-binding site, but fall short of assigning this to the transport site, which is currently thought to be in the middle of the membrane, based on the results of site-directed mutagenesis and secondary-structure predictions [31]. Mutagenesis has also been used to discredit the functionality of Ca$^{2+}$ binding to the stalk [32]. In terms of lanthanide binding, Girardet et al. [33] concluded that there were three classes of sites, and Squier et al. [34] suggested that lanthanides bind both to the stalk and to the putative transport sites in the middle of the membrane. Thus, it is difficult at this time to say whether the lanthanide sites observed by Asturias and Blasie [30] actually correspond to the Ca$^{2+}$-transport site, to a second, perhaps regulatory, Ca$^{2+}$-binding site, or to a site unique to lanthanides.

**Indirect structural studies: number of transmembrane helices**

In lieu of direct structural determinations, much energy has been devoted to predicting the folding of the amino acid sequence into secondary-structures. Figure 1 shows one model, which is based on a large number of considerations, including hydrophathy plots, detailed comparison of the ATP-binding domains with those of various soluble enzymes, more general secondary-structure predictions of other cytoplasmic domains, the overall shape of the cytoplasmic domain as determined by electron microscopy, spectroscopic and crosslinking studies, and sidedness of antibody binding. The number of predicted membrane crossings (hypothesized to be transmembrane helices) is hotly contested, with various groups arguing either for an even or for an odd number of crossings. Ovchinikov et al. [35] support the existence of only seven transmembrane helices for the Na$^+$/K$^+$-ATPase, but MacLennan, Green and co-workers claim 10 helices for Ca$^{2+}$-ATPase (series of papers beginning with [4]). MacLennan and Green might be willing to eliminate two of these helices (numbers 8 and 10 in Fig. 1), but not in the fashion suggested by Jorgensen and Andersen [36] or by Serrano [37], who combine M5 and M6, thereby putting a crucial interhelix loop (M7–M8 in Fig. 1) on the cytoplasmic side of the membrane.
A cytoplasmic location for both amino- and carboxy-termini has been reported by groups working on several different P-type pumps, thus supporting the existence of an even number of transmembrane helices. The amino-terminus of Ca²⁺-ATPase has long been thought to occur on the cytoplasmic side of the membrane [38], but Matthews et al. [39] have now shown that antibody binding to the carboxy-terminus does not require detergent and that the carboxy-terminus is therefore also cytoplasmic. The carboxy-terminus of H⁺/K⁺-ATPase has also been located on the cytoplasmic side of gastric mucosa (i.e. outside these vesicles) by iodinating tyrosine residues with lactoperoxidase (G Sachs, F Mercier, M Besacon and K Munson, personal communication); after digesting the iodinated molecule with carboxypeptidase Y, 28% of the label was released, which represents the expected number of tyrosine residues between the carboxy-terminus and the last transmembrane helix. As the gastric mucosa consists of tightly sealed vesicles, neither labelling nor proteolysis would have been possible if the carboxy-terminus had been luminal. Finally, Hennessey and Scarborough [40] have identified tryptic cleavage peptides from Neurepsora H⁺-ATPase and have concluded that both the amino- and carboxy-termini are cytoplasmic.

Of the two models with an even number of transmembrane helices, that of MacLennan and Green is supported by three reports of antibody binding. Two independent groups, using antibody labelling, have shown that Ca²⁺-ATPase residues 870-890 form a loop on the luminal surface of sarcoplasmic reticulum [41*,42*]. In both cases, binding of monoclonal antibodies was tested in both the presence and absence of detergent, and both groups found that detergent was required for interaction between the antibody and this loop. Furthermore, immunogold labelling has shown that an antibody to the same region in H⁺/K⁺-ATPase (residues 879-900) binds on the inside of gastric mucosa, which corresponds to the extracellular surface and topologically to the lumen of sarcoplasmic reticulum (G Sachs, P Mercier, M Besacon and K Munson, personal communication). Alternative models containing either eight [36,37] or seven [35] transmembrane helices appear to be in conflict with these results. However, both these models were conceived for Na⁺/K⁺-ATPase, leaving the possibility, seemingly unlikely considering sequence similarities, that its folding is distinctively different from other members of the family.

Conclusion

Structural information is helpful to protein chemists and to molecular biologists in providing a physical model on which to chart both their results and their speculation. In the case of P-type ATPases, there have been plenty of both, resulting in a model with an extraordinary level of structural detail (Fig 1). It is important to realize that electron microscopy and X-ray diffraction have provided only the basic shape of the cytoplasmic domain and the distribution of mass across the membrane; additional detail has been derived from secondary-structure predictions, sequence comparisons, site-specific labelling both with organic compounds and with antibodies, proteolysis and crosslinking, etc. This accumulation of data has provided fertile ground for the interpretation of future structures, which should encourage those of us involved in direct structure determinations. As for most membrane proteins, this work has progressed with frustrating slowness, but prospects now seem good for obtaining structures of Ca²⁺-ATPase, Na⁺/K⁺-ATPase and H⁺/K⁺-ATPase at a better resolution. By using frozen-hydrated microscopy, we may hope to preserve better the individual molecules and their crystalline lattice. In so doing, we should reveal the internal structure of the molecules, including their intramembranous domains. If, as expected, these structures show the size, shape and arrangement of the various domains, then it will be possible to attempt to assign the structural domains to the functional ones proposed in Fig. 1. This assignment would promote speculation regarding the domain interactions that ultimately must couple the hydrolysis of ATP to ion transport.

References and recommended reading

Papers of special interest, published within the annual period of review, have been highlighted as:

* of interest
** of outstanding interest


Improved crystallization conditions result in larger thinner crystals of detergent-dissolved Ca²⁺-ATPase. Their symmetry is determined by electron microscopy of negatively stained specimens, and a packing model is presented.


Rapid freezing of the thin three-dimensional crystals is used to preserve the excellent order intrinsic to crystals. Electron diffraction from these frozen-hydrated crystals is shown at 4Å resolution and a projection map at 6Å. Based on a comparison of projection maps of negatively stained and frozen-hydrated crystals, a proposal is made for the shape of the intramembranous domain.

Despite technical difficulties in interpreting a reconstruction based on a three-dimensional reconstruction is performed from a single crystal. The authors replace Ca\(^{2+}\) with lanthanide ions and use their absorption edge to directly visualize their location on the cylindrically symmetric profile structure of the Ca\(^{2+}\)-ATPase in scallop sarcoplasmic reticulum. The resulting location on the transmembrane domain, the authors propose that these six residues form the Ca\(^{2+}\) transport sites. This is an important result and it is worth spending time on the details of the reasoning.

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This paper is predominantly a review of several papers from this group describing changes in the electron-density profile in response to various conditions. The goal is to slow the reaction cycle in order to obtain higher resolution data of E\(_1\)\(\rightarrow\)P. Changes induced by these conditions have the opposite sign to changes previously attributed to the formation of E\(_1\)\(\rightarrow\)P.

The authors claim that asymmetry was strongest at the extracellular domain of the molecule.
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40. Hennessy JP, Scarborough GA: Direct Evidence for the Cytoplasmic Location of the NH2- and COOH-Terminal Ends of the Neurospora crassa Plasma Membrane H+-ATPase. J Biol Chem 1990, 265:532–537. H+-ATPase is reconstituted into proteoliposomes with 85% of their cytoplasmic domains facing out. Mild proteolysis releases small peptides from the amino terminus and, after a thorough proteolysis, a decapeptide is purified by high-performance liquid chromatography and identified as the carboxy-terminal by direct sequencing.

41. Matthews I, Sharma RP, Lee AG, East JM: Transmembranous Organization of (Ca2+-Mg2+)-ATPase from Sarcoplasmic Reticulum: Evidence for Lumenal Location of Residues 877–888. J Biol Chem 1990, 265:18737–18740. Antibody binding, both to intact Ca2+-ATPase and to proteolytic fragments, is used to support a model with 10 transmembrane helices. Detergent is required for binding to residues 877–888, leading to the conclusion that they are lumenal. Residues 877–888 represent a critical loop that is placed on the cytoplasmic side by models from several other groups.

42. Clarke DM, Loo TW, MacLennan DH: The Epitope for Monoclonal Antibody A20 (Amino Acids 870–890) is Located on the Lumenal Surface of the Ca2+-ATPase of Sarcoplasmic Reticulum. J Biol Chem 1990, 265:17405–17408. The binding sites of two antibodies are determined by expressing fragments of Ca2+-ATPase and testing each for antigenicity. Binding of these, as well as one additional, well characterized antibody, is tested both on intact sarcolemmal reticulum and on detergent-dissolved sarcoplasmic reticulum. Thus, the same conclusion obtained by Matthews et al. [39] is reached regarding the lumenal location of the loop between M7 and M8.