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**Structure of the calcium pump from sarcoplasmic reticulum at 8 Å resolution**

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The calcium pump from sarcoplasmic reticulum (Ca2+-ATPase) is typical of the large family of P-type cation pumps. These couple ATP hydrolysis with cation transport, generating cation gradients across membranes. Ca2+-ATPase specifically maintains the low cytoplasmic calcium concentration of resting muscle by pumping calcium into the sarcoplasmic reticulum; subsequent release is used to initiate contraction. No high-resolution structure of a P-type pump has yet been determined, although a 14-Å structure of Ca2+-ATPase, obtained by electron microscopy of frozen-hydrated, tubular crystals, showed a large cylindrical head connected to the transmembrane domain by a narrow stalk. We have now improved the resolution to 8 Å and can discern ten transmembrane α-helices, four of which continue into the stalk. On the basis of constraints from transmembrane topology, site-directed mutagenesis and disulphide crosslinking, we have made tentative assignments for these α-helices within the amino-acid sequence. A distinct cavity leads to the putative calcium-binding site, providing a plausible path for calcium release to the lumen of the sarcoplasmic reticulum.

As in previous studies, tubular crystals were induced in rabbit sarcoplasmic reticulum by using decavanadate. We also included an inhibitor, dapsyl thapsigargin, that promotes crystallization by

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locking Ca\textsuperscript{2+}-ATPase in the so-called E\textsubscript{2} conformation, which is characterized by low-affinity calcium binding from the lumenal side of the membrane\textsuperscript{6}. Tubes were imaged in the frozen-hydrated state, and optical diffraction of the best images revealed a strong layer line at 10.5-Å resolution. The narrowest tubes (600 Å in diameter) were selected for image processing, which involved a combination of Fourier space averaging and real-space averaging (see Methods). The phase residuals indicated that the resulting data extended to at least 8-Å resolution (Table 1).

The resulting map (Figs 1, 2) reveals many rod-like densities, separated by \(~\text{10 Å}\), that presumably correspond to \(\alpha\)-helices. Because the transmembrane and stalk domains are predicted to be entirely \(\alpha\)-helical (Fig. 3), we have concentrated exclusively on these regions. Initially, \(\alpha\)-carbon backbones were easily fitted to nine rod-like densities within the transmembrane domain (Fig. 2c–f), but this left a strong, unmatched density on the lumenal side of the membrane (arbitrarily labelled 'I' in Fig. 2f). Given the strong consensus for ten transmembrane helices\textsuperscript{7–9}, a tenth, highly inclined

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**Figure 2** Density cross-sections through the transmembrane and stalk of Ca\textsuperscript{2+}-ATPase. Four sections (2 Å thick) were superimposed in each panel (a–h) and their position is shown on the inset surface model. The top of the cavity is marked by an asterisk in e at the same location as in Fig.1c. The narrow passageway from the cytoplasm is marked with a cross in c. The lowest, dotted contour corresponds to 100% volume recovery; yellow, orange and red indicate increasing density.
helix was fitted to this last density. Unlike the initial nine helices, helix I does not traverse the membrane fully but meets helix E 6–8 Å from the cytoplasmic surface of the membrane (Fig. 2c, d). In general, only two of the ten helices (D and F) are inclined by <10°, five helices by 18° to 22°, and helices E and I by 27° and 34°, respectively, causing the relative positions of helices to change dramatically in sections parallel to the membrane plane (Fig. 2). Nevertheless, there are several clear groupings and a general trend toward right-handed twisting in these groups. In particular, pairwise associations occur between helices G and H, as well as between I and J, with the latter pair twisting around one another with a right-handed sense. A right-handed twist is seen again in the looser association between helices A and D and in the three-way association between helices B, E and F, at the cytoplasmic surface of the membrane, helix C is also associated with the latter group, but is excluded towards the luminal membrane surface. Between this threesome is a cavity (asterisk in Figs 1c, 2e) leading from the middle of the membrane to the luminal surface that is visible even at a contour level that includes 100% of the expected molecular volume (Fig. 1c) and appears to be surrounded exclusively by protein and traverses the hydrophobic core of the bilayer. The presence of such a cavity is consistent with a water-filled channel that could provide access from the calcium-binding sites to the lumen, as would be expected for the E2 conformation believed to populate this crystal form. Such a channel has previously been hypothesized to explain voltage effects on sodium binding to the E2 conformation of the related Na+/K+-ATPase, which suggest that cations move more than halfway across the membrane to reach their binding sites. Cytoplasmic access to the calcium sites should be restricted in the E2 conformation, but even so there is a potential passageway starting at the cytoplasmic surface between helices B, C, E and F (cross in Fig. 2c) and leading down to the beginning of the larger cavity (asterisk in Fig. 2c). This passage is visible only at a higher density cutoff, which is consistent with the restricted access expected for cytoplasmic calcium in the E2 conformation.

The stalk is 24 Å long and divided into four, rod-like densities, labelled α, β, γ and δ (Fig. 2b). The connection between these stalk densities and the transmembrane helices is obscured by the low contrast around the phosphate headgroups of the lipid bilayer (Fig. 2c). Nevertheless, the trajectory of α, β and δ suggests that they connect with transmembrane helices D, E and I, respectively; β and δ are almost collinear with E and I, whereas α is inclined at ~40° relative to D. According to structure predictions (Fig. 3a), the stalk is composed of four or five α-helices, and we found that α-helical backbones fit plausibly into α, β and δ densities. However, the fourth stalk density, γ, is smaller, has lower density throughout the stalk and a tenuous connection with transmembrane helix F; thus the identification of γ as a stalk helix and its association with F remains tentative. Densities Gp and Hp (Fig. 2b) form a raised platform on the cytoplasmic surface of the membrane directly above helices G and H; as seen in Fig. 1 (double arrowhead), these densities are set apart from the stalk and do not connect with the main cytoplasmic head, and so are less likely to connect transmembrane sequences with either of the two, large cytoplasmic loops (Fig. 3). A second, flat platform caps transmembrane helices A, B and C at the cytoplasmic membrane surface to the left of the stalk (single arrowhead in Fig. 1). On the luminal side of the membrane, a small, compact domain bridges helices I and J to a larger group containing helices B, C, E and F (Figs 1a and 2g, h). Helices G and H come together at the luminal surface and have only weak associations with surrounding densities, and so resemble a helical hairpin (Fig. 1b).

We then tried to associate transmembrane and stalk densities with helices predicted from the amino-acid sequence (Fig. 3). These predictions are supported by the current evidence for transmembrane topology and provide two important constraints in the form of direct connections between stalk helices and particular

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* Selection rule of the three independent data sets is characterized by the Bessel order \((n)\) of the (010) and (011) layer lines as defined in ref. 1.
† This data set, used for the final map, included 182 layer lines and was truncated at 8 Å resolution. It was derived from the real-space average by limiting the radial extent of each layer line to exclude noisy data with poor two-fold phase residuals. Numbers in parentheses indicate the percentage of data remaining after editing. Overall phase residual was 16.2°.
‡ Amplitude-weighted phase residuals for two-fold symmetry excluded equatorial data as well as data lower than 0.1% of the maximal off-equatorial amplitude (~99% of off-equatorial data were included). Random phases produce a phase residual of 45°.
transmembrane helices, and close proximity between several pairs of transmembrane helices connected by short loops. Further constraints come from the results of site-directed mutagenesis, which localized the calcium-binding sites between M4, M5 and M6 (refs 9, 13–16), and from disulphide links between pairs of cysteines introduced into M4 and M6 (ref. 17). The positions of the linked cysteines support a right-handed coiling of M4 and M6 over several helical turns. 

Residue variability can also shed light on helix packing, given the tendency for variable sites to face the lipid and for conserved sites to participate in helix–helix interactions18. We have extended a previous analysis19 by aligning transmembrane segments from several families of P-type pumps (Fig. 4a), using a few well-conserved markers within, or just outside, the hydrophobic segments and assuming an absence of gaps. The variable sites (Fig. 4b) of Na+/K+-ATPases (triangles) and plasma-membrane Ca2+-ATPases (circles) correspond well with those of SR Ca2+-ATPases (encircled letters), supporting the existence of a common transmembrane structure for the three families, even though there is less than 20% identity between the sequences of the transmembrane segments. 

In terms of helix packing, M4 and M6 are clearly the most highly conserved helices and should therefore have minimal exposure to bulk lipid. M5 is slightly less conserved, followed by M1, M2 and M8. M3, M9 and M10 are highly variable and the variable residues fall on one side of these helices, defining a face likely to be exposed to lipid. The specific positions of helices in Fig. 4b correspond to a section through the middle of the membrane at

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**Figure 4** Variability within the transmembrane sequences of P-type ion pumps. 
A. Sequence alignment between representatives of several subfamilies of P-type pumps: from the top, yeast H+-ATPase, rat H+/K+-ATPase, sheep Na+/K+-ATPase, rat plasma-membrane Ca2+-ATPase and rabbit sarcoplasmic-reticulum Ca2+-ATPase. These alignments were done according to refs 19, 25, but M8 for the PMCA and Na+/K+ families were realigned to match Q with E908 from SERCA126. Arrows define residues plotted in b; 1 and 2 indicate residues that contribute to calcium sites I and II9, respectively. 
B. Residue conservation was analysed within three subfamilies19 and variable residues are indicated by encircled letters for SERCA pumps, by filled circles for PMCA pumps, and by filled triangles for the Na+/K+-ATPase pumps. The helical wheels were superimposed on the density map (Fig. 2e) according to our most favoured assignment (Fig. 5a), and are oriented to expose variable faces to the lipid10 and to juxtapose calcium ligands8 (letters in triangles) and cysteine crosslinks17 in M4, M5 and M6. Letters correspond to the sequence of SERCA1; helices with normal letters are running away from the cavity, and those with mirrored letters towards the cytoplasm. The first residue in each helix is numbered at the centre and marked with a filled diamond; the first heptad along the helix is numbered (2–7) and linked to corresponding residues in successive heptads by the curved arrows.

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**Figure 5** Alternative sequence assignments for the transmembrane helices. 
Although we favour the assignments in a, the ambiguity in the connection between stalk and transmembrane helices could produce assignments like that in b, in which the cavity would still be surrounded by M4, M5 and M6. A more direct assignment of the N terminus to the raised platform is shown in c, but the long distance between M8 and M9 is inconsistent with their short connecting loop; also, the corresponding M9–M10 loop is inconsistent with the map. The assignments in d, suffer from the higher exposure of M4 to the lipid and from the large distance between M4 and M6 at the cytoplasmic side of the membrane, where disulphide crosslinking was most efficient17. M2, M3, M4 and M5 have been shaded to reflect their predicted connection to stalk helices. The small filled black circles correspond to the two bound calcium ions, and arrows emanate from residues thought to coordinate these ions (see Fig. 4b).
Unfortunately, these constraints and considerations are not sufficient to assign unambiguously the sequence of transmembrane helices in our map. Nevertheless, we favour one set of assignments (Figs. 4b and 5a) over several others (Fig. 5b–d), which together provide specific hypotheses to be tested by further mutagenesis and crosslinking studies. Our preference is based on the most likely connections between stalk helices and transmembrane helices, for example \( \alpha \rightarrow D, \beta \rightarrow E, \gamma \rightarrow F \) and \( \delta \rightarrow J \) (Fig. 3b). The connection between \( \gamma \) and \( F \) is the most tenuous and, if correct, would probably involve an unstructured region near the membrane surface. Transmembrane density \( E \) is most central and so has been associated with the most highly conserved, stalk-associated helix, M4. Densities B, E and F form a right-handed coiled-coil that surrounds the distinctive cavity, and have thus been assigned to M4, M5 and M6. The geometry of this arrangement (Fig. 4b) is consistent with the mutagenesis results that associate particular residues with either of the two calcium-binding sites and specifically places the C terminus at the side of the intersection of channels from the cytoplasmic and luminal sides of the membrane. Assignment of M8 to the highly inclined density I allows E908 to project into the latter channel, consistent with its effects on calcium transport.2,13 M9 would thus be rather short, perhaps owing to the unwinding of its polar carboxy terminus (NSLSEN; Fig. 4a). Density J is very exposed to lipid yet clearly extends into the stalk, making it a good candidate for the variable M3 helix. The extreme variabilily of M9 and M10 make this helical hairpin an attractive candidate for the isolated pair of transmembrane densities G and H, which are clearly associated at the luminal side of the membrane (Fig. 1b). Indeed, these assignments are consistent with the luminal disposition of several transmembrane densities (Fig. 2f–h), although the visualization of short loops at this resolution may in itself be unreliable. The loop between M7 and M8 is the longest, composing the bulk of the luminal domain and presenting an exposed, antigenic site,2,21 consistent with M7 and M8 being on opposite sides of the molecule.

An apparent discrepancy arises from putting the larger amino terminus in the flat platform and the smaller C terminus in the raised platform. This could be explained either by a disordered N terminus, or by putting the N terminus in the raised platform and hypothesizing a disordered or unstructured connection to M1; in the latter case, the arch of density connecting G with A in Fig. 2c could represent this connection.

Thus, we have resolved ten helices within the transmembrane domain of Ca\(^{2+}\)-ATPase and tentatively identified the site of calcium binding. Consistent with the conformation in this crystal form, a large cavity provides access to this site from the lumen, with a large cavity providing access to this site from the lumen, with a large cavity providing access to this site from the lumen, with a large cavity providing access to this site from the lumen, with a large cavity providing access to this site from the lumen, with a large cavity providing access to this site from the lumen, with a large cavity providing access to this site from the lumen, with a large cavity providing access to this site from the lumen, with a large cavity providing access to this site from the lumen, with a large cavity providing access to this site from the lumen. This brings an oblique overall path for calcium through the molecule, and structural studies of the alternative E1 conformation will be required to define the structural changes that drive transport along this path.

**Methods**

Crystals were prepared as described, except that 30 \( \mu M \) dansyl thapsigargin\(^3\) was added during crystallization. New strategies were used for processing images. To begin, a reference data set was compiled from the two best images in each of three symmetry groups (n = 6 for (0,1) layer line and n = 21, 22, –23 for (1,0) layer lines according to previous nomenclature)\(^1\) using established methods. Individual tubes were divided into short stretches (~1,000 \( \AA \)) and positional parameters refined by comparing Fourier data with data from the corresponding reference data set\(^2\). This procedure corrected slight stretching and bending of the tubes, led to improved phase statistics over previous methods, and allowed the use of longer areas along slightly bent tubes, improving signal-to-noise ratios significantly. Initial defocus levels were determined from Fourier amplitudes obtained either from nearly carbon film or from the tube itself\(^9\), and were later refined by comparing phases from the individual tubes with those in the averaged data sets. Data from each symmetry group were averaged and compensated for the contrast transfer function assuming 4.6% amplitude contrast\(^8\). These three independent data sets were edited to exclude noisy data, and were truncated at either 14- or 10-\( \AA \) resolution before calculating three-dimensional maps. After adjusting their relative magnifications, molecules within the three unit cells were masked and aligned in real space by cross-correlation; at this stage, twofold symmetry was not enforced and alignment parameters were determined independently for the twofold related molecules composing the unit cell. New maps were then created from a full set of unedited data to 6.5-\( \AA \) resolution, aligned according to these same alignment parameters, weighted according to the square root of the number of contributing molecules, and averaged in real space (Table 1). This averaged map was then used to generate a set of Fourier data with the helical symmetry of the alignment reference (n = 22 for (1,0) layer line), which were edited and truncated to 8-\( \AA \) resolution before calculating the final map.

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