Keeping calcium in its place: Ca^{2+}-ATPase and phospholamban

David L Stokes

Electron microscopy is gradually revealing more and more about the structure of the calcium pump from the sarcoplasmic reticulum, Ca^{2+}-ATPase. The most recent result reveals the ATP-binding site, and two different avenues are being pursued towards achieving a higher resolution structure. Although no such structures are currently available for phospholamban, various spectroscopies and site-directed mutagenesis have been combined to produce a compelling structural model for its regulation of Ca^{2+}-ATPase.

Addresses
Skirball Institute for Biomolecular Medicine, New York University Medical Center, 540 First Avenue, New York, NY 10012, USA; e-mail: stokes@saturn.med.nyu.edu

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Abbreviations

COMP	cartilage oligomeric matrix protein
EPR	electron paramagnetic resonance
FTIR	Fourier transform IR
IR	infrared
PAGE	polyacrylamide gel electrophoresis
PKA	cAMP-dependent protein kinase
PLB	phospholamban
SDS	sodium dodecyl sulfate

Introduction

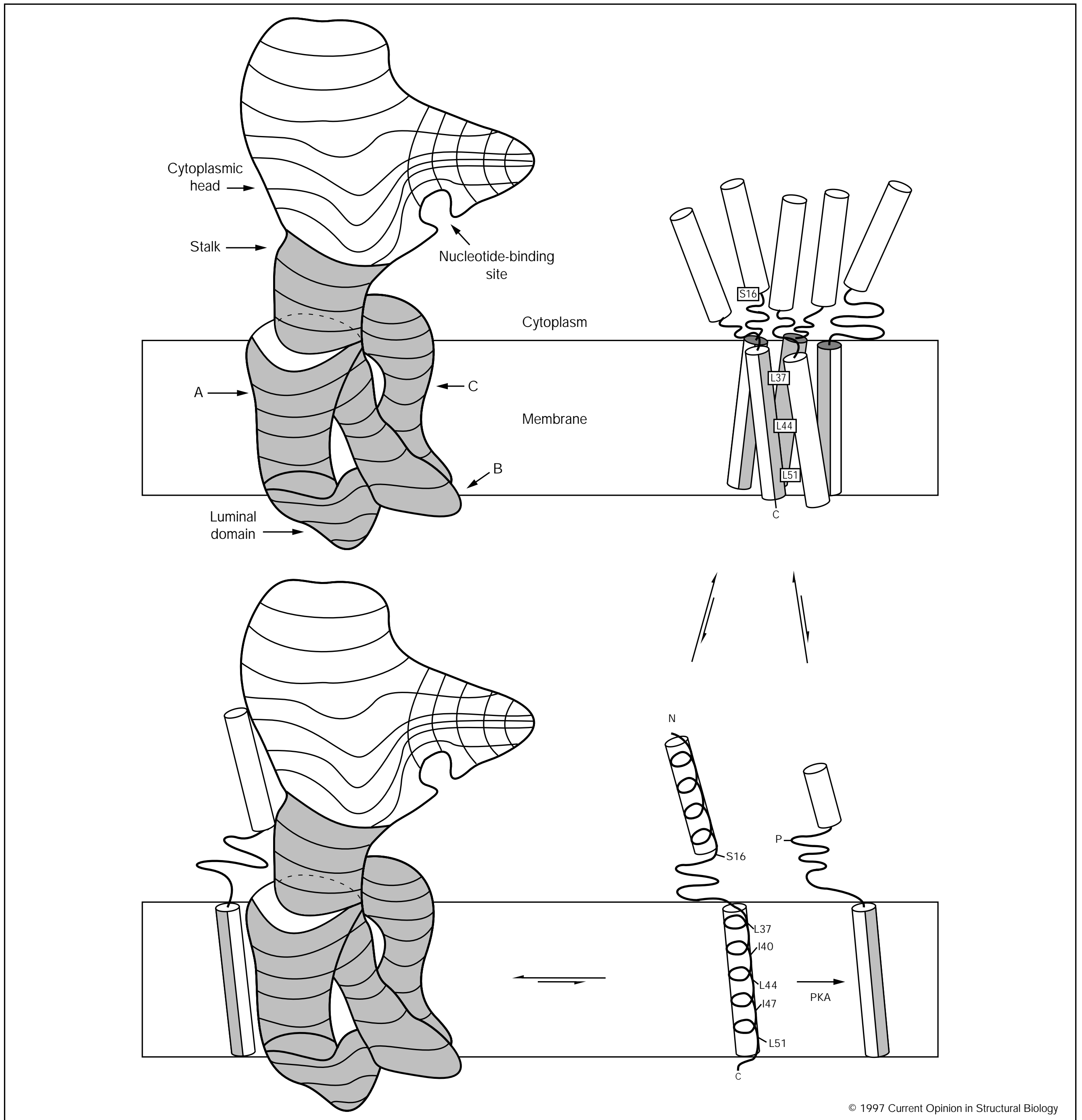
Calcium is used to signal a great many cellular functions, such as muscle contraction and synaptic transmission. Often, this calcium is released from internal stores (i.e. endoplasmic or sarcoplasmic reticulum) via calcium channels, and the patterns of release produce dramatic images of sparks and waves when visualized by confocal microscopy. A more mundane, but equally important role is filled by the ATP-dependent, transmembrane pumps that restore the low, resting levels of calcium within the cytoplasm. So reliable are these pumps, that an elevated calcium level is taken to signify cell death, thus initiating the mechanisms of apoptosis. This review will highlight recent progress on the structures of the calcium pump from sarcoplasmic reticulum, Ca^{2+}-ATPase, and of its regulatory protein, phospholamban (PLB). Ca^{2+}-ATPase is the dominant protein in the sarcoplasmic reticulum of all muscle cells and represents an archetype for a large family, called P-type ion pumps, that maintains ionic homeostasis in a wide variety of contexts. PLB interacts with Ca^{2+}-ATPase within the sarcoplasmic reticulum membrane and thus mediates an important regulation of cardiac contractility, which helps determine the response of the heart to exercise and to disease.

Tubular crystals of Ca^{2+}-ATPase

Large quantities of Ca^{2+}-ATPase can be derived from the sarcoplasmic reticulum of skeletal muscle and this material has been used for structural studies for over ten years. Several different crystal forms have been discovered, one of which has lead to 3D structures determined by electron microscopy [1,2]. These particular crystals are induced in the native sarcoplasmic reticulum membrane when Ca^{2+}-ATPase is locked in its calcium-free conformation (known as E_2) in the presence of the decameric form of vanadate. Both chelation of calcium by ethylene glycol-bis(β-aminoethyl ether) N,N,N′,N′-tetraacetic acid and incubation with the specific inhibitor thapsigargin invoke this E_2 conformation and thus promote crystallization. The resulting 2D membrane crystals take the form of membranous tubes, which can grow to be very long. Recent reconstructions have taken advantage of the helical symmetry, which is well preserved in frozen-hydrated tubes, to generate structures at 14 Å resolution [3,4••]. At this resolution, the boundaries of the bilayer are clearly visible and the transmembrane part of the molecule is easily distinguished from the cytoplasmic and luminal parts. The transmembrane domain is divided into three domains, which have been subsequently shown to accommodate the ten transmembrane helices that are hypothesized to span the membrane [5]. The cytoplasmic domain consists of a large head with a nose (or beak) that connects to the membrane via a narrow stalk, which is thought to comprise a bundle of four or five α helices [6]. The underside of the nose contains a large cleft that has been proposed as a possible nucleotide-binding site (Figure 1).

The assignment of this nucleotide-binding site has been recently confirmed by Yonekura *et al.* [4••] by comparing structures in the presence and absence of the nucleotide analog Cr-ATP. The most significant difference density has been found to be exactly in the cleft previously proposed as the nucleotide-binding site. To obtain this interesting result, Yonekura *et al.* have developed technologies for adding and subtracting structures in real space by masking, aligning, and scaling individual molecules from independent maps, in this case, those from tubes with and without Cr-ATP. In addition to revealing the position of Cr-ATP, their analysis has also determined the statistical significance of density differences, showing that the difference density assigned to Cr-ATP not only is the largest positive difference but also has the highest level of statistical significance (99.9% confidence level). Only a few, small differences are seen in other regions of the molecule, suggesting that Cr-ATP causes minimal conformational changes when bound to the calcium-free form of the pump (E_2). This is not expected to be true

Figure 1



Structures of Ca^{2+}-ATPase and PLB and a model for their interaction. The cartoon for Ca^{2+}-ATPase (top left) is based on 3D reconstructions at 14 Å resolution by cryoelectron microscopy [3]. A nucleotide analog, Cr-ATP, has been recently visualized in the nucleotide-binding site [4••] and the packing of α helices in the stalk and transmembrane domains should be revealed in a forthcoming structure at ~8 Å resolution. The pentameric structure of PLB (top right) consists of a five-stranded coiled coil in the transmembrane domain and an undetermined arrangement of N-terminal helices in the cytoplasm. Although the existence of this cytoplasmic helix is supported by NMR and FTIR spectroscopies, its orientation relative to the transmembrane domain is uncertain. Ser16 at the base of this cytoplasmic helix is the site of phosphorylation by PKA. Three leucines are labeled in the membrane domain and are part of the leucine zipper that stabilizes the coiled coil (shaded face of the cylinders). A dynamic equilibrium between the PLB pentamer and monomers exists within the membrane and it is the monomeric species (bottom right) that is now thought to interact with Ca^{2+}-ATPase. This interaction occurs at two sites, one in the cytoplasm and one in the membrane (bottom left); according to mutagenesis results, the face of PLBs transmembrane helix interacting with Ca^{2+}-ATPase (unshaded face) is opposite to the leucine zipper. Finally, phosphorylation of PLB by PKA shortens and destabilizes the cytoplasmic helix, disrupts the interaction with Ca^{2+}-ATPase, and favors the oligomeric form of PLB. Amino acids are labeled in single-letter code.

for ATP binding to the calcium-bound form ($E_1{\cdot}Ca_2$), which has been shown by various spectroscopic methods to involve major conformational changes.

These methods of real-space alignment will enhance the future of image reconstruction, not only by allowing visualization of ligands and labels, but also by removing a major bottleneck in averaging data. In particular, a significant inconvenience arises from the fact that these membranous tubes have variable helical symmetries, and, as a result, only a subset of tubes with matching symmetries could traditionally be used for Fourier-space averaging. These real-space methods allow averaging of data regardless of symmetry and have thus been employed to increase the number of images contributing to an average map. As a result, the resolution of data from Cr-ATP-bound Ca^{2+}-ATPase currently extends to 8Å resolution and the corresponding map contains columns of density that undoubtedly correspond to transmembrane and stalk helices (K Yonekura, C Toyoshima, personal communication). Thus, we will soon be treated to a direct look at some of secondary structure composing this molecule.

Multilamellar crystals of Ca^{2+}-ATPase

In addition to the tubular, 2D crystals grown in the sarcoplasmic reticulum membrane, 3D crystals can be grown from detergent-solubilized Ca^{2+}-ATPase. Unlike membrane protein crystals used previously for X-ray structures at atomic resolution, however, considerable amounts of lipid are present in the case of Ca^{2+}-ATPase and the crystals consist of bilayers, which are stacked into a multilamellar structure. This so-called 'Type I' morphology for membrane protein crystals is fairly common but is not generally suitable for X-ray work [7]. Nevertheless, these Ca^{2+}-ATPase crystals appear to be well ordered and the stacking can be controlled such that they are thin enough for electron microscopy. Initial work showed that electron diffraction could be recorded at ~4Å resolution from untilted crystals and a projection map has been presented at 6Å resolution [8]. In recent years, progress has been made in determining the geometry of stacking and in developing strategies for 3D reconstruction using this crystal form.

The first approach to determine the stacking geometry has been to examine very small crystals that offer a view parallel to the membrane plane [9•]. Earlier work had established a centered lattice within the bilayer and a twofold symmetry axis parallel to the membrane plane, thus placing these crystals in the C2 space group [10,11]; however, this space group makes no constraint on the geometry of stacking (i.e. distance between the lamellae and the angle between the *c* axis and the membrane plane). A big surprise from these small crystals has been that they adopt two different stacking schemes, depending on the amount of glycerol present during crystallization (5% versus 20%). At lower glycerol concentrations, the bilayers are closer together and a more extensive set of interlamellar contacts are formed, but packing within the bilayer is unaffected. The transition between these two schemes is discrete and, in both cases, the stacking is well ordered and phase relationships are consistent with the C2 space group. X-ray powder patterns have been used to verify the respective spacings and, furthermore, to indicate yet another stacking geometry for the large, thin, plate-like crystals used for electron diffraction; thus, new strategies will be required to determine the stacking geometry of these.

A projection map from these small, multilamellar crystals has been compared with an analogous projection from the tubular, vanadate-induced crystals to reveal structural changes that accompany calcium binding to Ca^{2+}-ATPase. This comparison follows from the notion that multilamellar crystals comprise the calcium-bound conformation of Ca^{2+}-ATPase, $E_1{\cdot}Ca_2$, whereas the calcium-free conformation, E_2 assembles into the tubular crystals [12]. As expected, major structural differences are apparent, particularly in the beak that contains the site of ATP binding. These differences are being considered in greater detail by another electron microscopy study of these small crystals at 8Å resolution (H Ogawa, DL Stokes, C Toyoshima, unpublished data). Following a quantitative comparison of the two projection structures, it is clear that the binding of calcium induces large-scale segmental movement in the molecule in addition to rearrangement of mass within the ATP-binding domain.

For 3D reconstruction, the large, thin crystals must be used ultimately, and substantial progress in this direction has been made both in crystal formation and in the analysis of electron diffraction patterns. The initial crystals were only 1–2 μm in diameter and diffracted weakly to 4Å resolution [8], but improvements in crystallization have been reported by Shi *et al.* [13]. Interestingly, glycerol has been again involved determining crystal size and morphology and, if manipulated judiciously, results in thin crystals with a diameter of 10–20 μm that diffract strongly to 3.5Å resolution. When tilted at small angles, diffraction spots are visible in electron diffraction patterns; these so-called Laue zones result from the sampling of diffraction amplitude normal to the lamellar plane. Such sampling does not occur if crystals are confined to a single layer or if the stacking of lamellae is disordered. Two different methods have been presented to determine the number of lamellae (unit cells) composing individual crystals. The first is based on the distribution of amplitude in these Laue zones and the second is based on crystal absorbance measured from optical densities in low magnification images of freeze-dried crystals. Thus, most crystals chosen for imaging are 3–5 unit cells thick (450–750Å), making them suitable objects for 3D reconstruction.

Recent work (D Shi, DL Stokes, unpublished data) has implemented strategies for merging diffraction data into a 3D data set. Several practical considerations prevent the use of methods already established for 2D crystals: namely the uncertain geometry of stacking and the sampling of Fourier data normal to the lamellar plane, which produces rapid changes in amplitude and thus requires a very precise determination of the tilt axis and tilt angle for a given diffraction pattern. To come to grips with these problems, 10–12 electron diffraction patterns have been recorded from each crystal at a series of incrementing tilt angles, thus forming a tilt series of data. This is similar to previous work with crotoxin [14] and is analogous to oscillation photography by X-ray crystallographers, except that the angles are discrete and that far fewer angles can be sampled using electron diffraction because of the much greater radiation damage. Nevertheless, a reciprocal lattice can be uniquely fitted to data from the tilt series and the unit cell parameters have thus been uniquely determined. By merging data from 18 independent tilt series, a moderate resolution (~8 Å) data set now includes tilts of up to almost 30° and expresses the expected twofold symmetry of the C2 space group. Data from larger tilt angles can now be incorporated, and similar strategies can be used to record electron micrographs, which will provide phases to go along with electron diffraction amplitudes and which will eventually allow the calculation of a 3D map.

A consensus structure for phospholamban

In cardiac muscle, calcium pumping across the sarcoplasmic reticulum is regulated by β-adrenergic stimulation, which is ultimately responsible for controlling cardiac contractility. PLB is a small, 52-residue transmembrane protein that mediates this regulation by controlling the rate at which Ca^{2+}-ATPase pumps calcium as follows. In resting muscle, PLB suppresses calcium pumping, probably via a direct interaction with Ca^{2+}-ATPase. β-adrenergic stimulation activates cAMP-dependent protein kinase (PKA), which phosphorylates PLB thus causing this interaction to be disrupted. As a result, Ca^{2+}-ATPase is no longer suppressed and the increased rate of calcium uptake into the sarcoplasmic reticulum not only speeds up relaxation, but also primes the muscle for stronger subsequent contractions. The specifics of the interaction between Ca^{2+}-ATPase and PLB are unknown, and the structure of PLB remains sketchy. Recent studies, however, have addressed the secondary structure of PLB as well as the structural basis for the pentameric form that is commonly adopted by PLB. Most importantly, a new model has evolved for the role of this pentamer in the regulation of Ca^{2+}-ATPase by PLB (Figure 1).

The first group of studies used either FTIR (Fourier transform infrared) or NMR spectroscopies to address the structure of the PLB monomer. Like circular dichroism, FTIR is able to determine the relative amounts of α helix, β sheet, and random coil; for these studies, however, PLB is reconstituted into lipid membranes that are deposited onto a flat surface, thus allowing the orientation of α helices to be probed with polarized IR radiation. Two independent studies have compared spectra from full-length PLB with those from the transmembrane domain and thus proposed specific arrangements for the secondary structure composing PLB. Despite large differences in the source of materials (expressed protein [15] versus synthetic protein [16]) and in the methods of specimen preparation (hydrated single bilayers [15] versus dried multilayers [16]), the results are reasonably similar. The transmembrane domain (residues 25/26–52) is predominantly helical (20–22 residues) and the helix is slightly inclined from the membrane normal (18–27°). Spectra from full-length PLB have been used to deduce the structure of the cytoplasmic domain, which is also predominantly α-helical (13–18 residues out of ~26). According to Tatulian *et al.* [15], *in situ* phosphorylation of Ser16 by PKA causes a decrease in α-helicity by five residues; however, Arkin *et al.* [16] find no change in conformation when their peptide is phosphorylated prior to purification and reconstitution. The resulting structural models differ mainly in the hypothetical location of the cytoplasmic helix. Arkin *et al.* [16] favor a helix contiguous with that from the transmembrane domain and Tatulian *et al.* [15] put the helix near the N terminus, which, as we shall see, is more consistent with an NMR structure for this domain.

Although NMR is capable of directly determining the structure of a molecule of this size, the solubility of membrane proteins presents a big problem. To get around this problem, Mortishire-Smith *et al.* [17] have studied the cytoplasmic domain (residues 1–25) of PLB and Maslennikov *et al.* [18] have used chloroform/methanol to render full-length PLB soluble. The latter study has confirmed the α-helical nature of the transmembrane domain, but the hydrophobic nature of the solvent precludes any conclusions about the cytoplasmic domain. The former study has suggested that membrane association helps stabilize the secondary structure of the cytoplasmic domain, as the 25-residue peptide is disordered unless 30% trifluoroethanol or 2% SDS is used as a solvent. The structure determined in 30% trifluoroethanol forms an α helix from residues 1–15 and is disordered between residues 18–25, which puts the phosphorylation site (Ser16) at the end of the helix; phosphorylation of Ser16 appears to disorder the helix after residue 12 and to reduce the intrinsic stability of the remaining helix. Despite the use of a nonphysiological solvent, the consistency of these results with those from FTIR makes this NMR structure plausible and supports Mortishire-Smith *et al.*'s [17] specific N-terminal location for this cytoplasmic helix in the intact molecule.

PLB forms a very stable pentamer that has been thoroughly characterized using SDS-PAGE. Several studies have recently addressed the structural basis for its stability. These studies have not employed any traditional

physical method but have systematically mutated every residue in the transmembrane helix and monitored the oligomeric state of the resulting protein using SDS-PAGE. This has resulted in a plausible model in which the transmembrane helices adopt a five-stranded coiled-coil. In particular, Engelman and coworkers [19] expressed chimeric constructs of the transmembrane domain of PLB linked to staphylococcal nuclease, which was an approach they had previously used to investigate dimerization by glycophorin. The fact that their chimeric protein also forms a stable pentamer by SDS-PAGE suggests that oligomerization of PLB is driven by its transmembrane domain. Examination of the mutants reveals a pattern in which mutation of every third or fourth amino acid disrupts the pentamer. This pattern is consistent with a five-stranded α-helical coiled-coil with a left-handed twist, which is further supported by the high proportion of α helix detected in this domain using FTIR. Molecular dynamics has been employed to select an atomic model with a minimum energy [19,20]. The theme of this group of studies has been to justify the formation of an ion channel by PLB. The physiological evidence for calcium conductance by PLB remains unconvincing, however, and the extremely hydrophobic nature of the 'pore' makes it difficult to imagine it being a conductance path for a charged ion.

Jones and coworkers [21••] have taken a similar approach to elucidate the pentamer structure, except they have expressed intact PLB rather than a chimeric construct. The observed effects of mutagenesis on pentamer stability are very much consistent with those of Engelman and coworkers [20]. Rather than using molecular dynamics, Jones and coworkers [21••] base their structural model on the formation of leucine zippers between adjacent helices, which are proposed to result in a left-handed, five-stranded coiled-coil. Although the overall structure is similar to that of Engelman and coworkers [20], the orientation of individual helices in the coiled coil differ by a 45° rotation around the helix axis, thus maintaining consistency with the structural formalism of coiled-coils [22]. Furthermore, Jones and coworkers' [21••] model is made credible by the recent X-ray structure [23•] of a structurally related — but functionally and evolutionarily unrelated — molecule from the extracellular matrix: cartilage oligomeric matrix protein (COMP). COMP comprises a very long five-stranded coiled-coil, which is stabilized by a leucine zipper that exactly matches that of PLB (i.e. leucines in the 'a' position of the heptad repeat and isoleucines in the 'd' position). Given this apparent structural homology, this structure of COMP might therefore be used as a starting point for a new set of molecular dynamics calculations for PLB.

Until very recently, these studies of oligomerization have been dogged by doubt as to the physiological significance of the pentamer. The fact that pentamers appear on SDS gels and are stable unless samples are boiled in SDS does not address their existence in the membrane or their relevance in the regulation of Ca^{2+}-ATPase. A very recent study of PLB, however, provides evidence for a dynamic oligomerization of PLB in the membrane [24•]. EPR has been employed to measure the ratio of boundary lipids (i.e. less mobile lipids) to bulk lipids, which has then been used to estimate the oligomeric state of PLB on geometrical grounds. Cornea *et al.* [24•] have found that the results for wild-type PLB are consistent with 3.5 PLB molecules per oligomer and that phosphorylation pushes this to 5. One of the destabilizing mutations, Leu37→Ala, which produces a monomer in SDS-PAGE, has boundary lipids that are indeed consistent with a monomer; phosphorylation pushes this mutant towards a dimer. Unpublished work by this same group has shown that if two pools of PLB are labeled with either fluorescent donors or acceptors, a mixing of these pools (i.e. a quenching of fluorescence) occurs following reconstitution in a membrane, thus supporting the notion that these oligomers are in dynamic equilibrium with monomers. Interestingly, this mixing does not occur in SDS (M Li, LR Reddy, DD Thomas, personal communication).

The last, most recent paper brings together many of these ideas into a plausible hypothesis for regulation. MacLennan and coworkers [25••] have repeated the systematic mutagenesis of the transmembrane domain of full-length PLB and have coexpressed the corresponding mutants with Ca^{2+}-ATPase. Thus, they have correlated the oligomeric state of PLB with the ability of mutants to regulate Ca^{2+}-ATPase. They have verified the pattern of leucine and isoleucine residues that stabilize the pentamer and, significantly, have found that monomeric mutants have enhanced regulatory effects on Ca^{2+}-ATPase (i.e. the mutants suppress the apparent calcium affinity more than wild-type PLB). When positioned on an α helix, these leucine and isoleucine residues lie on one face of the helix (i.e. the one facing other helices in the pentamer; shaded in Figure 1); changes to residues on the opposite face suppress regulatory effects on Ca^{2+}-ATPase. Thus, MacLennan and coworkers [25••] hypothesize that one face of this transmembrane helix is responsible for pentamer stabilization, whereas the other face interacts with Ca^{2+}-ATPase. Consistent with this hypothesis, double mutants, in which residues have been changed on both faces, result in monomers that are ineffective at regulating Ca^{2+}-ATPase. This same group have previously identified a short sequence in the cytoplasmic domain of Ca^{2+}-ATPase (near the phosphorylation site) that is required for regulation by PLB [26], and cross-linking has been observed between Lys3 of PLB and this same region of Ca^{2+}-ATPase [27]. These results suggest that there are, in fact, two sites of interaction between monomeric PLB and Ca^{2+}-ATPase, one within the membrane and one in the cytoplasm.

Conclusions

Considering the secondary structure of the PLB monomer, the probable structure of the pentamer, and the regulatory behavior of the various mutants, a plausible, structural model for PLB regulation has emerged (Figure 1). According to this model, the pentamer represents a storage form for PLB which is in dynamic equilibrium with a monomeric pool. These monomers interact with Ca^{2+}-ATPase via transmembrane helices and at a specific site in the cytoplasmic head. Phosphorylation of PLB leads to destabilization and partial unwinding of PLB's cytoplasmic helix at the phosphorylation site; this structural change and/or the electrostatic effects of the added phosphate group disrupt the interaction with Ca^{2+}-ATPase and push the PLB population back towards pentamers. Still missing is a structure of full-length PLB, either alone or together with Ca^{2+}-ATPase; however, cryoelectron microscopy is currently underway in our laboratory on tubular crystals containing Ca^{2+}-ATPase and PLB (HS Young, DL Stokes, unpublished data), and we hope that soon we will have visual evidence to confirm this model and to elaborate on the structural basis of the regulation.

For Ca^{2+}-ATPase, structures at 8 Å resolution will be determined from tubular crystals in the near future and further specific sites will be located (e.g. the thapsigargin-binding site; P Zhang, D Stokes, unpublished data). In the slightly longer term, a higher resolution structure should be obtained from the multilamellar crystals, which should not only define secondary structure better, but also elucidate the conformational changes that accompany calcium binding. Thus, even in the absence of X-ray crystallographic studies, there is real promise for developing a structural understanding of ATP-dependent calcium transport.

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