LIPID-PROTEIN INTERACTIONS AND THE FUNCTION OF THE CA^{2+}-ATPASE OF SARCOPLASMIC RETICULUM

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I. INTRODUCTION

The sarcoplasmic reticulum (SR) of skeletal muscle cells is an intracellular membrane compartment that stores calcium in resting conditions. Following stimulation of the muscle cell, calcium is rapidly released into the cytoplasm, allowing actin-myosin interaction and muscle contraction.[1] Relaxation is effected by the action of the Ca-pump of SR, which couples the hydrolysis of ATP to the translocation of calcium back into the SR lumen.

The Ca-pump of SR is a Ca^{2+}-ATPase that has the ability to transport calcium against a large chemical gradient. It lowers the cytoplasmatic concentration of calcium to about 10^{-7} *M*, and has the capacity to pump calcium into the vesicular lumen until its concentration reaches the millimolar level. Part of the calcium inside the SR is free, and part is bound to intravesicular proteins.[1]

The Ca-pump of SR is one of the best known membrane enzymes. Since the early studies of Ebashi and Lipman[2] and of Hasselbach and Makinose,[3] who developed methods to isolate functional SR vesicles from skeletal muscle, a large number of studies dealing with different aspects of SR structure and function have been carried out. There is substantial experimental evidence regarding the kinetics of the Ca-ATPase reaction, with some insights into how the enzyme undergoes conformational changes during the cycle.[4-6] There are a number of studies on how lipid-protein interactions modulate the function of the enzyme, and it has been proposed that lipids have a specific role in the enzyme mechanism. However, other investigators hold the view that lipids solely provide a permeability barrier for the transported calcium, since many of the kinetic properties of the Ca^{2+}-ATPase are retained by the enzyme solubilized in detergents.

This review attempts to discuss the role of lipid-protein interactions in the function of the Ca^{2+}-ATPase. The experimental evidence supporting a role of lipids in enzyme function will be discussed in detail, and will be compared with the studies carried out with soluble enzyme preparations. While some investigators hold the view that the

enzyme solubilized with detergents retains all the properties of the membrane-bound enzyme, there is substantial evidence indicating that lipids have a role in enzyme function that goes beyond merely providing a convenient permeability barrier for the transported calcium.

II. STRUCTURAL ASPECTS

A. Structure of SR Vesicles

The SR in the muscle cells has different structural regions. At the triads, two terminal cisternae of SR (junctional SR) are linked by structures known as "feet" to the transverse tubules.[7] The terminal cisternae are continuous with the longitudinal SR (free SR), which is formed by narrow tubules that surround the myofibrils.[7] The entire SR compartment is isolated by the SR membrane from the cytoplasm and from the external medium.

Following homogenization of muscle cells, a microsomal fraction enriched in SR vesicles can be obtained by differential centrifugation. All the SR vesicles that are sealed after isolation have the same orientation, with the cytoplasmic side out. Further purification of SR vesicles has been obtained by centrifugation in density gradients.[8-10] The heavy SR fraction banding at higher densities contains calsequestrin,[8] an intravesicular protein with a large number of low-affinity calcium-binding sites.[8,11-13] The light SR vesicles, that do not contain calsequestrin and are composed mainly of Ca-ATPase protein, are derived from the longitudinal SR; the heavy SR is derived from vesiculation of the terminal cisternae.[8] Recent studies have described the isolation of a vesicular fraction enriched in terminal cisternae,[14] and it has been shown that the region of the SR membrane that forms the triads with the transverse tubules, the junctional membrane, has a different structure than the rest of the SR membrane and is devoid of Ca-ATPase molecules.[14,15] These studies indicate that the Ca^{2+}-ATPase is distributed evenly along the SR surface, except in the junctional region.

Structural studies of the isolated SR vesicles using electron microscopy, X-ray diffraction, and freeze-fracture techniques, indicate that the SR membrane is asymmetric, with a high density of particles protruding at least 5 nm in length from the membrane into the cytoplasm, but with no protrusions into the SR lumen.[16-25] These surface particles represent monomers of ATPase molecules, and are susceptible to tryptic digestion. Comparison with sequenced portions of the molecule indicate that two short and one long segment of the ATPase extend into the cytoplasm.[26] Furthermore, structural studies[27-33] show that the ATPase monomer has the dimensions of a cylinder, with an aqueous portion 40 to 60 Å in diameter 40 Å in length, and an intramembranous portion 40 Å in diameter and 40 Å in length.

Freeze-fracture studies of the isolated SR vesicles have shown that the density of intramembranous particles is lower than that of the surface particles.[20,21,34] It has been proposed that intramembranous particles represent aggregates of three to four ATPase monomers. Addition of vanadate can induce crystallization of the Ca-ATPase in SR membranes isolated from rabbit muscle, with a dimeric organization.[28-30,32,33] According to the studies of Taylor et al.,[33] the dimers are connected by a bridge at a height of about 4.2 nm above the bilayer surface. Additional interactions between monomers might occur in the regions of the molecule inserted in the lipid bilayer, but they would not be detected with the techniques used by these authors. Furthermore, native SR isolated from scallop muscle presents orderly aggregates of CA-ATPase dimers.[31,32] SR vesicles isolated from rabbit and frog muscle under similar conditions to those used to isolate SR from scallop muscle also show some areas with regular patterns,[35] although it could not be decided whether monomers or dimers were present.

Other structural studies[36] have shown that native SR membranes isolated from a variety of sources (rabbit, rat, guinea pig, frog) are covered by a disorderly array of dots that correspond to ATPase monomers, but small oligomers are seen (dimers, tetramers, and a few larger aggregates). Only very small lipid patches were seen in all vesicles. From these results, it was concluded that there are 31,000 to 34,000 ATPase monomers per square micron of SR surface. The same report[36] concludes that the density of the large intramembranous particles, seen in freeze-fractured SR, is consistent with the existence of tetramers predominantly, but some other aggregates are present.

Irradiation inactivation experiments[37-39] indicate that the minimum functional unit of the Ca^{2+}-ATPase in the membrane is a dimer. Consistent with this proposition are results obtained from X-ray and neutron diffraction data by Napolitano et al.,[27] who concluded that the intramembranous particles represent dimers. Kinetic experiments also indicate that the functional unit of the enzyme is a dimer.[4-6] However, this point is not unambiguously established yet since other results are consistent with the enzyme acting as a monomer during the entire reaction cycle (see Section IV).

It is important to point out that recent studies[40] have shown that, in the presence of Ca^{2+} or lanthanide ions, two-dimensional crystalline arrays of Ca^{2+}-ATPase molecules are formed. These arrays are developed by individual Ca^{2+}-ATPase monomers, and are different from the dimers formed by vanadate. The possible significance of these observations in showing different conformational states of the enzyme should be confirmed by other methods.

The complete amino-acid sequence of the Ca^{2+}-ATPase has been determined using complementary DNA techniques.[41] From the sequence, a model for the protein with three cytoplasmic domains joined to a set of ten transmembrane helices by a narrow, pentahelical stalk, was proposed[41] (see Figure 1). In addition, from this proposed structure possible regions of the molecule that participate in the calcium transport reaction were suggested[41] (see Section III, Reaction Mechanism).

B. Structure of SR Lipids

As discussed above, the Ca^{2+}-ATPase is the predominant intrinsic membrane protein present in the SR membrane (except in the junctional region). There are 80 to 100 molecules of phospholipid associated with one molecule of enzyme in the native SR membrane. Structural studies[42] indicate that these phospholipids are somewhat asymmetrically distributed between the two halves of the bilayer, with more phospholipid (54%) in the inner monolayer. In addition, these studies indicate that the average fatty-acyl chain extension in the inner monolayer is about 20% larger than in the outer monolayer.[42] It was suggested that this structural asymmetry of the phospholipids is caused by the presence of the Ca^{2+}-ATPase in the bilayer.[42]

Phospholipids are the main lipid components of the native SR membrane.[43-46] Phosphatidylcholine is the main phospholipid species (65 to 75%), followed by phosphatidylethanolamine (15 to 20%), and phosphatidylinositol (8 to 10%).[47,48] Other minor phospholipid species reported to be present in native SR vesicles probably reflect contamination with other muscle membranes.[49] Cholesterol is present only in low concentrations (5% or less).[46,50]

The fatty-acyl composition of the phospholipids present in SR (containing a mixture of heavy and light vesicles) is characterized by a number of highly unsaturated fatty acids, and by the presence of a significant amount of plasmalogens[44,45,47,51-53] (see Table 1). Both phosphatidylcholine and phosphatidylethanolamine contain unsatured fatty acids or aldehydes in the sn-2 position,[53] but it is clearly apparent that the major species (69%) present in phosphatidylcholine are 16:0 to 18:2 and 18:0 to 18:2, while

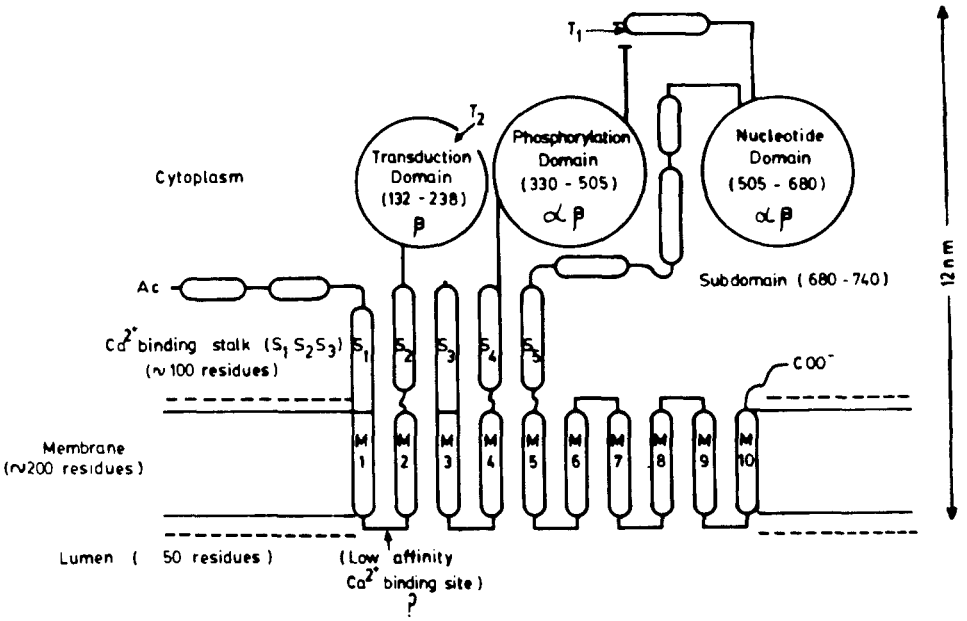

FIGURE 1. Schematic diagram of the assembly of the ATPase.[41] On the cytoplasmic side protrude three main domains, connected by five stalks (S_1—S_5) to the transmembrane domains (M—M_{10}). It has been proposed[41] that the high-affinity calcium-binding sites are located in stalks S_1 to S_3, whereas the low-affinity calcium-binding sites would be entirely different and presumably located on the luminal side of the M_1—M_2 loop. T_1 and T_2 represent the first and second tryptic cleavage sites, respectively. While this is a planar diagram, it is likely that in the native vesicles the ATPase molecule would be folded, with formation of tight clusters among the α-helical regions.[41]

Table 1
MOLECULAR SPECIES OF PHOSPHATIDYLCHOLINE AND PHOSPHATIDYLETHANOLAMINE IN SARCOPLASMIC RETICULUM ISOLATED FROM SKELETAL MUSCLE

Species (sn-1—sn-2)	Phosphatidylcholine (%)	Phosphatidylethanolamine (%)
16:0—18:1	5.8—8.0	—
16:0—18:2	49.5—55.1	—
16:0—18:2 (P)	4.3—1.3	—
16:0—20:4	4.9—5.5	4.6—4.5
16:0—20:4 (P)	7.2—7.1	26.2—27.3
16:0—22:4 (P)	—	6.8—5.7
18:0—18:1	4.8—3.0	—
18:0—18:2	19.1—15.1	—
18:0—18:2 (P)	—	2.3—2.6
18:0—20:4	1.6—2.2	12.2—12.6
18:0—20:4 (P)	—	23.2—25.5
18:0—22.4 (P)	—	3.3—2.7
18:1—18:1	0.7—0.2	—
18:1—18:2	0.9—1.8	—
18:1—20.4 (P)	1.2—0.9	19.0—17.0
18:1—22:4 (P)	—	2.6—2.1

Note: Data were taken from Reference 53. The two values given for each species represent results obtained by different methods.[53]

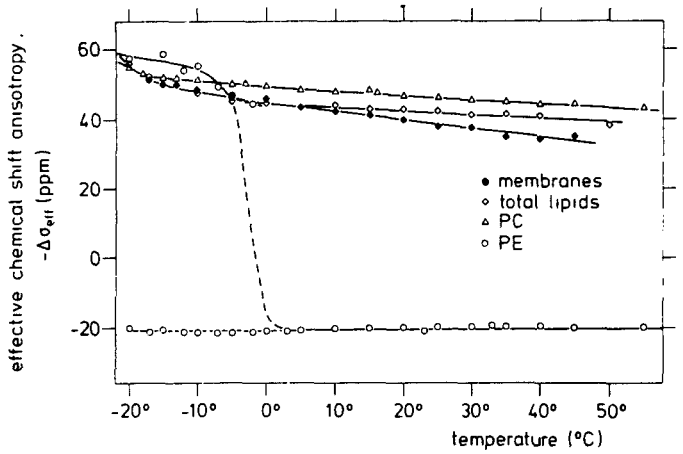

FIGURE 2. Temperature dependence of the effective ^{31}P chemical shift anisotropy, $\Delta\sigma_{eff}$, of sarcoplasmic reticulum membranes and the extracted and purified membrane lipids dispersed in 0.1 *M* KCl 10 m*M* Hepes 1 m*M* EDTA pH 7.0 (○) membranes; (◆) total lipid extract; (△) phosphatidylcholine lipid fraction; (◇) phosphatidylethanolamine lipid fraction (two coexisting components are seen with the latter at low temperature, indicating bilayer and hexagonal H_{II} structures).

69% of the species present in phosphatidylethanolamine are 16:0 to 20:4 (P), 18:0 to 20:4 (P), and 18:1 to 20:4 (P) (P meaning plasmalogen). This fatty-acid composition shows that phosphatidylethanolamine is considerably more unsaturated than phosphatidylcholine, and that the major species present in phosphatidylethanolamine are in the form of plasmalogens. This chemical composition of the phosphatidylethanolamine present in SR (high plasmalogen content with abundance of tetraenoic side chains at the sn-2 position) would result in stabilization of hexagonal II phase.[53] ^{31}P NMR experiments[54] and our own studies (Figure 2) show that phosphatidylethanolamine extracted from native SR vesicles undergoes a lamellar to hexagonal II phase transition at −10°C. It is likely that this transition arises from tetraenoic ethanolamine plasmalogens.[53]

The chemical composition of the individual sn-1 and -2 fatty-acyl chains of phosphatidylinositol in SR is not known. However, it has been reported that this phospholipid contains about 37% arachidonic acid (20:4), with a total content of 47% unsaturated fatty acids.[52]

While heavy and light SR have the same overall phospholipid composition,[55] the light SR lipid fraction has less unsaturated phospholipids (28 to 30%) than heavy SR[55] (48 to 49%). The fatty-acyl species present in each individual phospholipid of light and heavy SR remain to be determined.

The phospholipid species present in SR are asymmetrically distributed in the two halves of the bilayer. Thus, 70 to 80% of the phosphatidylethanolamine is present in the outer half (cytoplasmic side) of the bilayer.[56-60] In contrast, phosphatidylcholine seems to be either symmetrically distributed[61,62] or present a slight enrichment[60] in the inner monolayer (52 to 53%). Phosphatidylinositol, as determined by phospholipase digestion of SR vesicles, is mainly present (88%) in the inner monolayer.[60]

The overall picture of the SR lipid phase indicates that the outer monolayer contains less phospholipids than the inner monolayer, and that the outer monolayer is highly enriched in phosphatidylethanolamine. Since phosphatidylethanolamine contains mainly highly unsaturated plasmalogens in the sn-2 position, it is likely that the outer

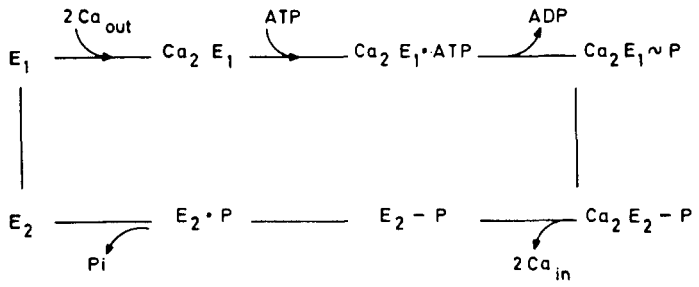

FIGURE 3. Postulated reaction cycle of the sarcoplasmic reticulum Ca^{2+}-ATPase. Only the essential features of the cycle are indicated. For further details, see text.

SR monolayer is more unsaturated than the inner monolayer, although this prediction has to be tested experimentally.

Physical studies carried out either with the native SR membrane or with reconstituted enzyme systems indicate that the SR phospholipids are present in two distinguishable motional populations, as probed with fatty-acid or phospholipid spin labels.[63-68] Reports describing two populations of phospholipids using ^{31}P NMR techniques[69] have been recently questioned[70] since only a single phase of the SR membrane lipids is detected by ^{31}P NMR spectroscopy of the native SR membrane.[70,71]

The spin-label probes report one component with similar motion to that obtained from extracted SR lipids, and another less mobile component (boundary lipids) that is presumably due to contact with the protein. The possible functional significance of this phospholipid distribution in the function of the Ca^{2+}-ATPase will be discussed below.

III. REACTION MECHANISM

The current picture of the reaction whereby the Ca^{2+}-ATPase of SR couples the energy derived from ATP hydrolysis to translocate calcium across the membrane is shown in Figure 3. This reaction scheme, originally proposed by de Meis and Vianna,[72] can be divided in the following steps:

1. The enzyme has two possible conformations: a form with high affinity for calcium (E_1), and a form with low affinity for calcium (E_2). E_1 has the calcium binding sites outside, and E_2 inside the vesicles.
2. Addition of calcium in the 10^{-6} *M* range causes the E_1 form of the enzyme to bind calcium. Two calcium ions are bound per enzyme molecule.
3. Subsequent addition of ATP allows enzyme phosphorylation. There is one phosphorylation site per enzyme.
4. The phosphoenzyme intermediate $E_1 \sim P$ undergoes a conformational change to E_2-P that results in a 1000-fold decrease in affinity for calcium. Only the phosphoenzyme form with high affinity for calcium, $E_1 \sim P$, can form ATP following addition of ADP. The translocation of calcium to the inside of the vesicular lumen is coupled to this conformational change from $E_1 \sim P$ to E_2-P.
5. Calcium dissociates from E_2-P and is released to the intravesicular side.
6. E_2-P decomposes to E_2 + Pi. The latter is released to the extravesicular medium.
7. The enzyme undergoes a conformational change from E_2 to E_1, and the cycle is completed. E_2, in the presence of Mg^{2+} and in the absence of calcium, can react with Pi to form E_2-P.

This overall reaction mechanism is supported by numerous experimental observations. Thus, calcium binding, in the absence of substrate, has been measured at equilibrium using ^{45}Ca;[73-76] currently, there is general agreement that there are two high-affinity calcium binding sites (Kd 10^{-6} *M*) per ATP site,[5] although some experiments indicate that at low temperatures there is only one high-affinity calcium binding site per ATP site[74,76] (for an opposing view, see Reference 77). Calcium binding is cooperative and shows competition with protons.[78,79] Following binding of calcium, the enzyme undergoes a conformational change that can be detected by changes in (1) intrinsic tryptophan fluorescence,[80-82] (2) in fluorescence of labels attached to sulfhydryl groups,[83] (3) in spectral properties of spin labels attached covalently to the enzyme,[84] (4) in sulfhydryl reactivity,[85,86] (5) in circular dichroism spectra,[87] and (6) in Fourier-transform infrared spectra.[88] The current evidence indicates that the two calcium ions are bound sequentially, and that there are two high-affinity calcium sites per enzyme. (For a detailed discussion see Reference 5.) Furthermore, fluorescence quench studies indicate that the change in tryptophan fluorescence induced by addition of calcium to the enzyme involves trytophan residues that are located in the hydrophobic part of the ATPase molecule, and thus in contact with the hydrocarbon chains of the membrane phospholipids.[89]

Addition of micromolar concentrations of ATP to the enzyme equilibrated with calcium (so that the two high affinity sites are occupied) results in the formation of a "high energy" phosphoenzyme intermediate, $Ca_2E_1\sim P$,[90-94] by transfer of the terminal phosphate of ATP to an aspartyl residue localized in the catalytic site of the enzyme.[93,94] Concomitant with the formation of $Ca_2E_1\sim P$ the two calcium ions are occluded.[77,95,96] Addition of ADP can reverse the phosphorylation reaction, resulting in formation of ATP and release of Ca to the extravesicular solution. Only the $Ca_2E_1\sim P$ form has the capacity to occlude two calcium ions,[97] and to react with ADP to form ATP. Associated with the enzyme phosphorylation by ATP, conformational changes have been demonstrated by several different methods. Thus, ATP addition increases the α-helical content of the SR proteins,[88] while addition of calcium has the opposite effect and increases random coil conformations. An increase in β-antiparallel structure following ATP addition was also suggested.[88] Furthermore, addition of ATP to the ATPase covalently labeled with fluorescent probes causes an increase in fluorescence intensity[82,98] that correlates with phosphoenzyme formation. Changes in fluorescence energy transfer between ATPase molecules have also been detected following formation of phosphoenzyme either with ATP or with Pi as substrates.[99] In contrast, no changes were detected in ellipticity of the membrane-bound enzyme either by addition of calcium or by the formation of the phosphoenzyme intermediate with Pi, since identical circular dichroism spectra were obtained in all conditions.[87] It was concluded from these results that major reorganizations of secondary structure do not take place during the enzyme reaction mechanism.[87]

Kinetic studies have revealed that $Ca_2E_1\sim P$ (ADP sensitive) is transformed into an ADP-insensitive intermediate,[100,101] E_2-P, with much lower affinity (Kd 1 m*M*) for calcium. Following this conformational transition from $Ca_2E_1\sim P$ to E_2-P, calcium is released from the enzyme, as has been shown by several investigators.[74,102,103] In the native SR vesicles, calcium would be released into the intravesicular space[104] until its concentration reached the millimolar range, whereby the dissociation of calcium from E_2-P would stop. Following calcium dissociation, E_2-P would react with water producing Pi and E_2.

The entire reaction cycle of the Ca^{2+}-ATPase is reversible. Thus, it is possible to form E_2-P from Pi at low pH in the presence of Mg^{2+} and in the absence of calcium (see Reference 105 and references therein). The same aspartyl residue phosphorylated

by ATP is phosphorylated by Pi.[106] The interaction of the enzyme with Pi is favored by the addition of dimethyl sulfoxide, which lowers the concentration of Pi required to form E_2-P (from 10 m*M* to 10 μM) and abolishes the pH dependence (between 6 and 7) of the phosphorylation reaction.[104] It has been proposed[107,108] that the formation of E_2-P from Pi requires dissociation of water molecules, and that the phosphate group in E_2-P might reside in a pocket of the protein structure with a relatively nonaqueous environment. Direct structural evidence in favor of this hypothesis is needed to ascertain its validity.

The transition from the $Ca_2E_1\sim P$ to the E_2-P intermediate is crucial to the calcium transport mechanism.[4-6,72,109] The coupling rules proposed by Jencks[110] establish that only $Ca_2E_1\sim P$ can react with ADP to form ATP, and only E_2-P can react with water to give Pi. The reversible transitions from $Ca_2E_1\sim P$ to E_2-P can take place only when the high-affinity sites are occupied by calcium. E_2, but not E_1, can react with Pi to form E_2-P; conversely, only Ca_2E_1 can react with ATP to form $Ca_2E_1\sim P$. From these coupling rules it follows necessarily that the stoichiometry of the transport system is fixed: two calcium ions are transported per ATP hydrolyzed. However, other stoichiometries have been found,[111] suggesting that these coupling rules are not absolute.

The crucial question regarding the coupling mechanism is understanding what are the conformational changes that allow calcium to be translocated to the vesicular interior, and how these changes are made possible by the conversion of the $Ca_2E_1\sim P$ intermediate to the E_2-P form.

Some evidence has been building up regarding this matter. Thus, it is now known that there is one catalytic ATP site per enzyme,[112] a finding that has clarified the reaction mechanism, and that makes unlikely, for example, models of coupling involving half-site stoichiometry. It is now clear that the pump is electrogenic,[113-116] that Mg^{2+} ions are probably not countertransported with Ca^{2+} ions,[117,118] and that protons might be pumped in opposite direction to calcium ions during transport.[117,119] Recent structural studies show that the distances between the two high-affinity calcium sites of the enzyme is about 11 Å, indicating that the two sites are close to each other.[120,121] In contrast, the ATP catalytic site is ~35 Å from the two calcium sites, indicating that the Ca^{2+} sites and the nucleotide site are quite farther apart in the three-dimensional structure of the enzyme.[120,121] Studies using fluoresceinyl isothiocyanate (FITC) as a fluorescent ATP analog indicate that the nucleotide catalytic site of the enzyme is partially protected from the bulk solvent.[122] After binding of calcium to the high affinity sites, the degree of exposure of FITC to the solvent increases, as determined by the increased quenching of FITC fluorescence caused by iodide.[122] These results show that despite the fact that the calcium sites and the nucleotide site are not contiguous, calcium binding induces long-range conformational changes that affect the nucleotide site.

The model proposed by MacLennan et al.,[41] based on the complete amino acid sequence of the enzyme, assumes that all three extramembranous domains of the enzyme are highly interactive (see Figure 1). The nucleotide domain (residues 505 to 680) would be relatively independent of the rest of the molecule, but would have to interact with the phosphorylation domain (residues 330 to 505) to transfer the phosphate to the aspartyl residue. Following phosphorylation, the transduction domain (residues 132 to 238) would change conformation allowing calcium translocation.

Since the structural studies of Nakamoto and Inesi[87] rule out major changes in secondary structure of the protein during the reaction mechanism, it is likely that enzyme transitions take place by reorientation of protein segments. In order to account for the long-range conformational effect of phosphorylation on calcium affinity (the two sites are far apart[120,121]), it has been proposed that residues 132 to 238 participate in the

long-range conformational effect.[87] Twisting and rearrangement of the α-helices forming the stalks in the model of MacLennan et al.[41] produced by phosphorylation, might result in a change in the microenvironment of the bound calcium, decreasing its affinity.[87] Furthermore, it has been reported that the E_1 and the E_2 enzyme conformations, as well as the respective phosphorylated intermediates Ca_2E_1P and E_2-P, are associated with different protein structures, as evidenced by their different tryptic digestion patterns,[123] both in the membrane-bound enzyme and in detergent-solubilized monomers.

IV. DETERGENT-SOLUBILIZED ENZYME

It has been shown that the Ca^{2+}-ATPase can be solubilized in detergents, such as Triton X-100®, deoxycholate, or dodecyloctaethylene glmonoether ($C_{12}E_8$), with full retention of ATPase activity.[124-140] Additional studies have shown that the monomeric enzyme, obtained by $C_{12}E_8$ solubilization, can carry out most of the partial reaction steps illustrated in Figure 3. Thus, the monomer has the ability to undergo conformational changes following calcium binding to the two high affinity sites, as detected by fluorescence changes.[141,142] It has been reported that while two calciums are bound per enzyme monomer in a cooperative fashion,[134,143] only one calcium is bound per monomer when the enzyme is in dimeric form, with loss of calcium binding cooperativity.[143] The monomer can be phosphorylated by ATP in the presence of calcium.[135,144-148] Although it was initially reported that the monomeric enzyme had lost the ability to release calcium following phosphorylation,[149] recent studies indicate that the monomer undergoes the same changes in calcium affinity associated with the conformational transition from $Ca_2E_1\sim P$ to E_2-P as does the membrane-bound enzyme[141] and that the soluble monomer undergoes the same structural changes during the reaction cycle as the membrane-bound enzyme.[142] These combined observations have been used to suggest that the overall reaction cycle can be carried out by a monomer of the Ca^{2+}-ATPase.[6] However, the monomeric soluble enzyme shows some differences with regard to the membrane-bound enzyme,[130,131,144-146,150,151] with the most striking difference being its marked instability in the absence of calcium. Thus, in contrast to the behavior of the membrane-bound enzyme, the solubilized monomer undergoes rapid and irreversible inactivation in calcium-free solutions.[129,134,135,140,147] These results indicate that the membrane configuration is needed to protect the enzyme from inactivation in the absence of calcium. It is likely that in the soluble monomer some conformational changes take place in calcium-free solutions (i.e., by exposure of regions normally buried in the lipid bilayer) that cause irreversible denaturation. From this observation, it follows that the lipid bilayer is needed at least to maintain the enzyme in stable conformations throughout the reaction cycle.[152]

Another important difference in the behavior of the solubilized enzyme with regard to the membrane-bound enzyme is that following solubilization, the reverse reaction, i.e., phosphorylation of the enzyme by Pi, is lost[135,140,146] although there are results reporting enzyme phosphorylation by Pi with properties similar to these of the membrane-bound enzyme.[133,153]

Both the stability of the solubilized enzyme in the absence of calcium and the ability to form phosphorylated enzyme by Pi are recovered by addition of glycerol or dimethylsulfoxide.[135,140] Furthermore, there are contradictory reports on the ability of the soluble enzyme, phosphorylated by Pi, to transfer the phosphate to ADP to form ATP. Thus, little or no ATP formation has been reported for the enzyme solubilized with Triton X-100®, unless dimethylsulfoxide is added to the reaction solution.[140] However, another report describes that the monomeric enzyme, solubilized with $C_{12}E_8$, is phosphorylated with Pi in the presence of 30% dimethylsulfoxide, and retains the

ability to form ATP after ADP addition even at low concentrations of dimethylsulfoxide.[135]

It has been shown that at low ATP concentrations the solubilized enzyme has a twofold faster phosphoenzyme turnover than the membrane-bound enzyme; increasing ATP concentration to the millimolar range yields identical phosphoenzyme turnover for both preparations.[135] Moreover, while oligomeric ATPase presents the same complex dependence on ATP concentration as the membrane-bound enzyme, monomeric ATPase has a monophasic dependence on ATP concentration.[103,130,131,143,146]

These results suggest that the solubilized enzyme, presumably in monomeric form, carries out the partial reactions of the membrane-bound enzyme, but with additional requirements (e.g., cosolvents), or at different rates. Thus, the differences exhibited by the monomeric enzyme might reflect either loss of protein-protein associations, such as those present in an oligomer, or reflect perturbations in the hydrophobic environment surrounding the monomers (the detergent molecules do not have the same structural arrangement as the native phospholipid bilayer). A combination of both effects might also be involved, and this point remains to be determined. It is interesting to note in this regard that no changes in aggregational state have been observed during the ATPase reaction cycle carried out by the monomer in detergent solution,[133,148] indicating that the monomer can carry out the entire cycle. However, other structural studies using radiation inactivation analysis have shown that the target size of the membrane-bound enzyme corresponds to a dimer, and that this target size does not change following enzyme phosphorylation either by ATP or by Pi.[38] These results suggest that during the reaction cycle the enzyme does not dissociate from its dimeric form. Furthermore, small-zone chromatography of the soluble ATPase showed that addition of ATP displaced the monomer-dimer equilibrium towards the monomer.[143] However, the results obtained with soluble enzyme systems cannot be readily extrapolated to the SR membrane, with its very high density of ATPase molecules. In this context, it has been suggested that if during ATP hydrolysis there are changes in protein association, it is likely that these changes weaken interactions between subunits rather than produce complete monomerization of the protein.[154]

V. LIPID-PROTEIN INTERACTIONS

A. General Considerations

Although there are numerous studies on the role of lipids on SR function, few studies exist regarding the metabolism of SR lipids. It has been shown in this context that the synthesis of SR lipids is not closely coupled to the synthesis of the SR membrane proteins[155] and that isolated SR vesicles have the capacity for phospholipid synthesis.[156] Furthermore, the presence of phospholipid methyltransferase activity has been demonstrated to be highly localized in SR, showing that phospholipid methylation of PE can result in PC formation in isolated SR vesicles.[157] In addition to these studies, recent experiments indicate that isolated SR vesicles have the kinase that phosphorylates phosphatidylinositol to phosphatidylinositol-4-monophosphate.[158,159] The physiological roles (if any) of both phospholipid methylation and phosphorylation are not clear at present.

Since the early studies of Martonosi,[160] it has been known that lipids have a role in the function of the Ca^{2+}-ATPase. Thus, removal of endogenous lipids or modification of the physical state of the lipids around the enzyme affect the ATPase activity of SR vesicles.[49,161] Furthermore, it has been proposed that specific lipids might have a role in coupling Ca^{2+}-transport to ATPase activity, as will be discussed later in the text.

B. Role of Lipids in Ca^{2+}-ATPase Activity

1. Delipidation Experiments

There are many studies describing inhibition of ATPase activity after removal of membrane lipids.[160,162-168]

Removal of a substantial fraction of the endogenous lipids present in SR vesicles, from the original 90 to 100 lipids per enzyme down to 40 to 30 lipids per enzyme, has no effect on Ca^{2+}-ATPase activity.[165,168] This point is now well established, despite a couple of reports describing inhibition of ATPase activity following only partial removal of lipids.[63,169]

Subsequent removal of lipids below 40 to 30 lipids per enzyme causes a drastic inhibition of ATPase activity, to almost undetectable levels for preparations containing 10 to 15 lipids per enzyme. The inhibition can be reversed by readdition of lipids.[168]

Parallel determinations of steady-state phosphoenzyme formation by ATP as a function of lipid content have shown that this reaction proceeds normally if the lipid content decreases from 90 to 100 lipids per enzyme to about 22 lipids per enzyme.[168] Inhibition of steady-state phosphoenzyme formation was observed only when the lipid content decreased from 22 lipids per enzyme to below 10 lipids per enzyme[168] (Figure 4). The inhibition in this case can also be reversed by readdition of lipids, provided 14 or more lipids remained associated with the enzyme. It is important to point out that the evidence available so far indicates that the phospholipid composition does not change as a function of lipid content,[166,168,169] so that even highly delipidated preparations of ATPase retain the same phospholipid composition as the native SR vesicles.

The results discussed above indicate that while phospholipids are required to sustain ATPase activity, different steps in the enzyme reaction cycle have different sensitivities towards delipidation, with phosphoenzyme formation being more resistant to delipidation than phosphoenzyme decomposition. These new findings reconcile apparent discrepancies found in the literature, since depending on the extent of lipid removal, inhibition solely of phosphoenzyme decomposition[43,163,166] or of both decomposition and formation[51,167] will be observed.

Lipid removal does not affect other partial steps of the ATPase reaction cycle, such as calcium and nucleotide binding, but markedly interferes with binding of vanadate.[170,171] The inhibition can be reversed by addition of oleic acid. Furthermore, while in native SR vesicles vanadate displaces calcium from the high-affinity sites and is displaced by binding of calcium to the low-affinity sites, in lipid-deprived preparations these reactions do not take place.[170,171] Furthermore, the lipid-deprived preparations form only very limited amounts of the E_2-P intermediate following reaction with Pi.[167] These results indicate that the reverse reaction (phosphorylation by Pi) is very sensitive to lipid removal, a finding that might reflect the requirements of this reaction for a suitable hydrophobic environment.[108]

2. Lipid-Replacement Experiments

The development of procedures[172,173] to replace the endogenous SR lipids with well-defined chemical species has prompted many studies to determine the effect of changing the lipid composition on the function of the Ca^{2+}-ATPase.

It has been clearly established that fluid lipids are needed to support ATPase activity (for a review, see Reference 49). A variety of experiments carried out with the enzyme reconstituted in synthetic saturated phospholipids have shown that the ATPase activity is drastically decreased when the lipids surrounding the enzyme are in the gel phase.[64,166,173-178] The activity can be readily recovered either by warming the system above the gel transition temperature,[64,166,173,175-178] or by addition of detergents.[175] However, in several cases the enzymatic activity markedly increases at temperatures

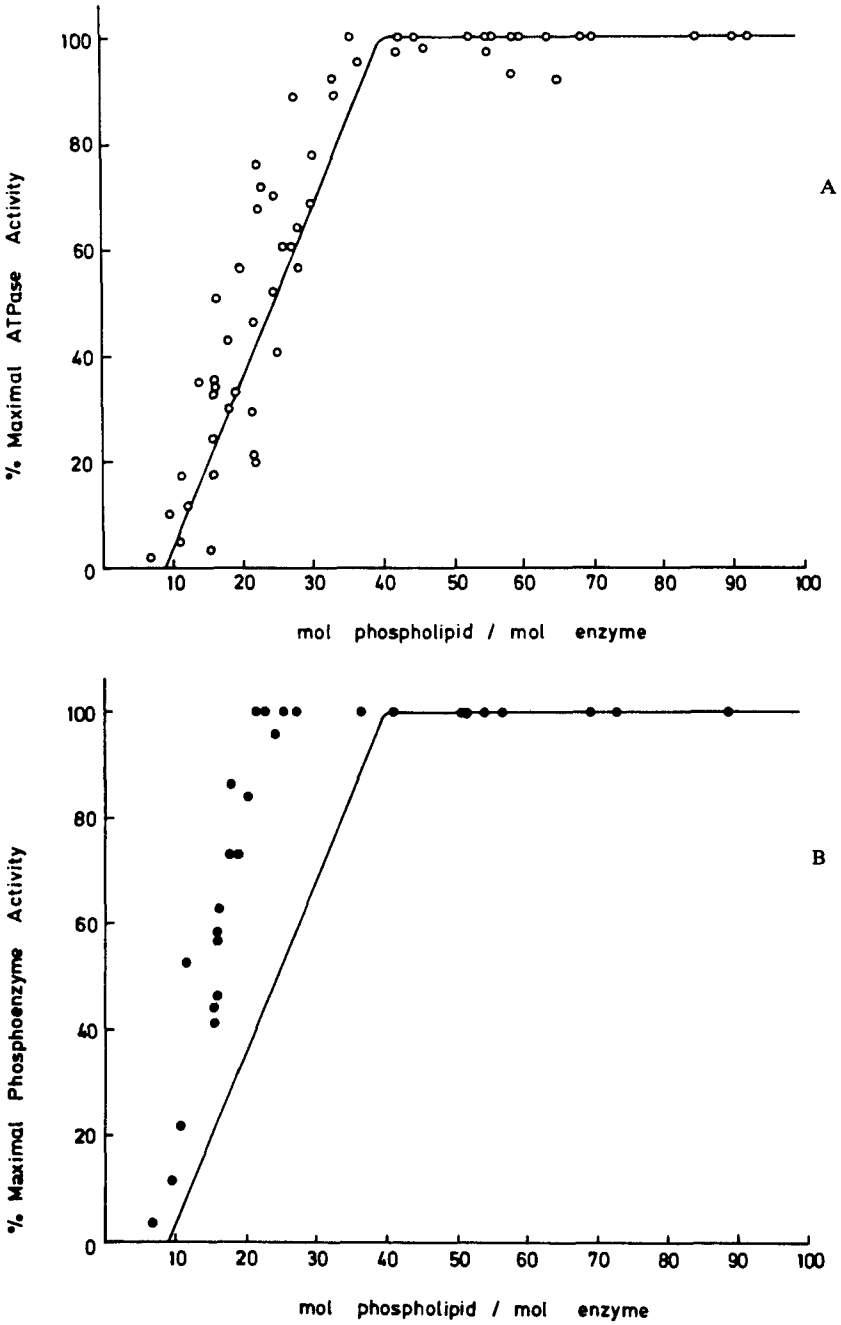

FIGURE 4. (A) ATPase activity as a function of lipid content. The mol phospholipid per mol of enzyme was calculated assuming a molecular weight of 115,000 for the Ca^{2+}-ATPase enzyme, and a protein content of 90% ATPase in all preparations (as determined by gel electrophoresis in sodium dodecylsulfate-containing polyacrylamide gels). The solid curve represents the rotational mobility of the enzyme, giving as 100% the maximal value of mobility observed, measured as a function of lipid content using saturation transfer EPR. (The data of rotational mobility as a function of lipid content were taken from Squier, T. C., Ph.D. thesis, University of Minnesota. The values were scaled to the same protein content as described above.) (B) Phosphoenzyme formation as a function of lipid content. Conditions are the same as those described in (A), and the solid curve represents the % change in protein rotational mobility as a function of lipid content. Conditions are the same as those described in (A).

lower than the transition temperature of the synthetic lipid around the enzyme, indicating that the protein perturbs the lipid phase (for a detailed discussion of this point, see Reference 49).

In analogy with the observations made on delipidated preparations, our studies indicate that the presence of gel-phase lipids around the enzyme causes inhibition of ATPase activity by selectively blocking phosphoenzyme decomposition with no effect on the enzyme phosphorylation by ATP.[166,175] Furthermore, the phosphoenzyme intermediate formed is presumably $Ca_2E_1 \sim P$, since it is ADP sensitive at low temperatures, and does not change with time to the ADP-insensitive form.[166] However, there are other reports describing inhibition of phosphoenzyme formation by ATP for enzyme surrounded by gel-phase lipids.[176,177] The reasons for this discrepancy are not known.

Synthetic lipids in the gel phase cause a drastic decrease in the rotational mobility of the protein that correlates with the inhibition of ATPase activity (see below). Whether the inhibition of rotational mobility is caused by the decreased fluidity of the lipid phase when lipids are in the gel state,[175] or whether it is caused by formation of protein patches excluded from the gel-phase lipids,[179-182] is a matter for discussion. However, other studies[173] show that the protein remains dispersed in gel-phase lipids, suggesting that the decrease in overall membrane fluidity is responsible for the inhibition of both protein rotational mobility and ATPase activity.

Lipid-replaced preparations containing either phosphatidylcholine or phosphatidylserine (negative membrane surface charge) show the same calcium-binding properties,[183] when measured in the presence of 10 m*M* $MgCl_2$ and 80 m*M* KCl. Removing magnesium or lowering the monovalent cation concentration increased the apparent association constant for calcium in both systems.[183] These results indicate that the calcium-binding domain is probably shielded from the surface charge of the phospholipid bilayer.

On the other hand, ATPase preparations reconstituted with saturated phospholipids at low temperatures have a remarkable capacity to occlude calcium ions, since long incubations in calcium-free solutions are needed to effectively remove calcium from the enzyme.[175]

3. Other Lipid Structural Requirements

In addition to a requirement for a minimal number of lipids in the fluid state, an optimal bilayer thickness is needed to sustain ATPase activity.[177,184,185] Furthermore, phospholipids (or glycolipids) but not cholesterol can support ATPase activity. Addition of cholesterol to reconstituted enzyme systems produces inhibition of ATPase activity, provided cholesterol is allowed to interact directly with the enzyme.[186] It is likely that the rigid cholesterol ring does not suit the structural requirements of the hydrophobic regions of the polypeptide chain.[68]

C. Boundary Lipids

As discussed in Section I, physical measurements using lipid spin labels indicate that a fraction of the 90 to 100 molecules of phospholipid per enzyme molecule present in SR vesicles, the boundary lipids or "annulus", are motionally more restricted than lipids in the bilayer configuration.[63-68] A more immobilized lipid component has also been detected with lipid spin labels covalently attached to the Ca^{2+}-ATPase.[187,188] Increasing temperature or addition of detergent decreased the fraction of the immobile component, a finding that was interpreted as evidence that the immobilized lipids were trapped in protein oligomers that would dissociate following detergent addition or increase in temperature.[187,188] However, it has been shown[189] that covalently attached long-chain spin labels explore more than just the layer of lipid adjacent to the protein.

Thus, the decrease in the fraction of the immobile component following addition of detergent or increase in temperature could well reflect increased exchange of the label between the boundary and the adjacent layers, instead of dissociation of presumptive oligomers.

Other studies (for a detailed discussion, see Reference 49) using different physical techniques have also shown that the SR lipids exist in two different populations.[169,173,190,191] Furthermore, it has been proposed[173,191] that the lipids beyond the boundary layer, the "secondary domain", are less ordered than lipids in the bilayer configuration. There are conflicting results regarding the mobility of the lipids in this secondary domain, since it has been reported that they are either motionally more restricted[66] or less restricted[67] than bilayer lipids in the absence of protein.

In addition to the two populations of lipids present in SR vesicles, it has been proposed that the Ca^{2+}-ATPase has extraannular binding sites for lipids, such as cholesterol[192] or fatty acids.[193,194] The significance of these extraannular binding sites on enzyme function remains to be established.

It was proposed initially that the lipids forming the annulus exchanged very slowly with bulk lipids,[64] but NMR experiments[71,195-198] have established a lower limit for the exchange rate, which is several orders of magnitude higher than the turnover rate of the enzyme. These findings have brought into question the role of boundary lipids in modulating enzyme function.[199] However, the boundary lipids could exchange rapidly but still maintain the enzyme in a given conformation, provided that once in contact with the protein they changed to the structure needed for optimal enzyme function.

Regarding a role of boundary lipids in enzyme function, it was proposed by Hesketh et al.[64] that full occupancy of an annulus of 30 lipids around the enzyme was needed for maximal ATPase activity. This proposal was based on the fact that they observed inhibition of ATPase activity only after delipidation to less than 30 lipids per enzyme, plus their spectroscopic data showing a less mobile lipid component that became preponderant below 30 lipids per enzyme.[64] Most other workers, however, have reported lower numbers for the boundary lipid component of SR — 20 to 25 lipid molecules per enzyme monomer.[63,65,68] If we accept this latter number of lipids as forming the boundary lipid component, it is interesting to note that inhibition of ATPase activity starts to take place at about 40 lipids per enzyme,[168,200] a higher number of lipids per enzyme than has been reported for the boundary lipid component (Figure 4). However, inhibition of enzyme phosphorylation by ATP is observed when lipid is decreased below 22 lipids per enzyme,[168] the same number of lipids as those forming the boundary layer. These results indicate that the boundary lipids are probably needed to maintain the enzyme in a configuration dispersed enough to allow access of ATP either to its binding site or to the catalytic site, or access of calcium to the high affinity binding sites (see Figure 1). According to this view, removal of lipids from the boundary layer might promote protein aggregates or might induce conformational changes that would hinder the accessibility of ligands (ATP or calcium) to the enzyme, thus preventing phosphoenzyme formation.

The selective inhibition of ATPase activity observed in the range of 22 to 40 endogenous lipids per enzyme[168] indicates that removal of lipids in this range interferes in a reaction step subsequent to phosphorylation of the enzyme by ATP. Physical studies measuring the overall rotational motion of the Ca-ATPase reconstituted with variable amounts of endogenous lipids have shown[200] that partial removal of lipids, down to 40 lipids per enzyme, has no effect on the enzyme-rotational mobility (see Figure 4). Below 40 lipids per enzyme, however, there is a linear decrease in overall protein rotational mobility to 15% of the values obtained with enzyme preparations containing 40 to 90 lipids per enzyme.[200] Furthermore, a linear correlation between the decrease in

protein mobility and the inhibition of ATPase activity was observed,[200] suggesting that the Ca-ATPase needs the lipid environment provided by 40 lipids in order to undergo the rotational mobility needed to carry out the forward reaction cycle at normal rates.

The postulated conformational changes of the enzyme that take place following the phosphorylation reaction but prior to phosphoenzyme decomposition (see Section III), are likely to be inhibited under conditions that substantially reduce the rotational mobility of the enzyme. Previous results showing that a certain degree of rotational mobility is needed for optimal ATPase activity but not for phosphoenzyme formation[175] support this proposal.

From this overall picture, it is likely that rotational mobility is required to allow the enzyme to carry out the conformational changes needed to translocate calcium following phosphorylation by ATP, and that a minimum number of lipids, greater than the boundary lipid component, provides a suitable environment around the enzyme that would allow these conformational changes. In support of this proposal are recent studies showing that addition of diethylether to the SR membranes activates about twofold the ATPase activity, and fluidizes the SR membrane,[201] as detected by an increase in lipid hydrocarbon chain dynamics probed at several depths within the membrane bilayer. Both the bulk lipid and the boundary lipids are fluidized by ether, and there is a concomitant increase in protein rotational mobility[201] (about twofold). The increase in lipid fluidity produced by ether addition is equivalent to the effect of increasing the temperature of the system by about 10°C. These results indicate that increasing the fluidity of the boundary layer lipids produces an increase in protein rotational mobility that is reflected in an increased enzyme turnover and hence higher Ca^{2+}-ATPase activity.[201]

D. Effects of Temperature

1. Effect of Temperature on ATPase Activity

Many different laboratories have reported a break at 15 to 20°C in Arrhenius plots of ATPase activity, either in native SR vesicles, in leaky preparations of purified ATPase, or in SR vesicles made permeable to calcium by addition of the ionophore A23187.[166,202-210] Furthermore, the reverse reactions (synthesis of ATP, and ADP-induced calcium efflux) studied as a function of temperature[211] show breaks in Arrhenius plots at about 18°C. These results indicate that both the forward and the reverse reactions of the Ca^{2+}-ATPase cycle undergo a change at about 18°C, although the activation energies below 18°C are much higher[211] for the reverse reaction (45 to 50 kcal/mol) than for the forward reaction[49] (30 kcal/mol). Above 18°C the activation energies for both reactions are similar (17 to 20 kcal/mol).[49,211]

Different explanations have been proposed to account for this break, including lipid phase transitions[166,202] or conformational changes in the protein.[207-209] The finding that the enzyme solubilized in $C_{12}E_8$ also displays a break in activity at 20°C was interpreted as evidence of a change in enzyme conformation at this temperature.[129] However, further studies revealed that the break temperature is also affected by the nature of the detergent used to solubilize the enzyme.[132] Thus, the break in enzymatic activity not only reflects enzyme conformational changes, but most likely it is also modulated by the nature of the hydrophobic environment around the enzyme.[132]

Studies of ATPase activity as a function of temperature in enzyme reconstituted with synthetic fluid phospholipids have yielded conflicting results. Thus, the ATPase reconstituted in dioleoylphosphatidylcholine, a synthetic lipid that is in the liquid-crystalline state above −20°C, has been reported to produce linear Arrhenius plots with no breaks,[166] nonlinear plots,[174] or breaks at 30[203] or 18°C.[208] From these results it is not possible to conclude that the break at 20°C is solely due to a conformational change in the enzyme independent of the lipid phase.

2. Effect of Temperature on SR Lipids

Physical studies on the effect of temperature on the properties of the SR lipids have also yielded contradictory results. Breaks in Arrhenius plots of spectral parameters for nitroxide spin labels incorporated into the SR membrane have been reported to take place at 18 to 20°C,[64,166,202,212] suggesting a change in the lipid phase (phase transition) at these temperatures. However, the ESR spectra of lipid spin labels contain components in the slow-motion regime even in the fluid phase.[213] This fact has not been taken into account in the spectral studies carried out with SR membranes. Therefore, the temperature dependencies described might contain artifactual breaks, especially when the effective parameters used are more strongly affected by the amplitude of motion, as is the case with order parameters, than by the rate of motion (correlation times).

Other studies with spin-label lipid probes incorporated in SR have reported no breaks in spectral parameters for the bilayer lipids in the 4 to 40°C range,[214] but a clear break in the boundary lipid component at about 15°C (covalently bound long-chain spin labels were used to probe the boundary lipid region). These findings are in agreement with the studies of Heremans and Wuytack,[215] who, based on the effect of pressure on the break temperature of ATPase activity in SR vesicles, suggested a change in the boundary lipid region at 20°C.

In addition to lipid spin labels, fluorescent probes have been used to detect changes in the SR lipids with temperature. Breaks in spectral parameters have been observed at 18[205] or 20°C.[216]

It has been established that the lipids in the SR membrane are in the fluid (liquid-crystalline) configuration at temperatures higher than 10°C,[217] or even at lower temperatures,[206] 1 to 5°C. These findings exclude a phase transition of the SR lipids at 20°C as the cause of the breaks in Arrhenius plots of ATPase activity.

Recent studies[210,218] have described the temperature dependence of the fluidity of the SR lipids, extracted from the membrane, using nitroxide lipid probes. It was found that the SR lipids present linear Arrhenius plots for spectral parameters of probes that report on different depths in the bilayer. The presence of protein decreased the average hydrocarbon chain mobility, but linear Arrhenius plots were found for the fluidity of SR lipids forming part of the native SR vesicles as well. These results definitely rule out a major change in lipid fluidity at 20°C, that could explain the break of ATPase activity at this temperature.

Other studies[219] using transparinaric acid as a fluorescent probe incorporated in the SR membrane have shown that there are changes in the environment around the ATPase at 15°C as detected by a decrease in fluorescence energy transfer between the enzyme tryptophan residues and the probe above 15°C. In addition,[219] the susceptibility of the enzyme to tryptic digestion, measured as a function of temperature, shows an increase at 15 to 20°C. These results were interpreted in terms of a change in the microenvironment around the enzyme at 15 to 20°C, that might be correlated with the temperature-dependent behavior of its ATPase activity.

Other studies, using different probes to quench intrinsic trypophan fluorescence, have shown that the ATPase selects fluid lipid over gel-phase lipids and that quenching is more efficient when the enzyme is surrounded by fluid lipids.[220-223]

All these combined observations suggest a change in the lipids surrounding the enzyme at 20°C that might modulate the ATPase activity, but the physical basis for this change remains to be established.

3. Effect of Temperature on the Rotational Mobility of the Enzyme

The rotational mobility of the Ca^{2+}-ATPase either in the native SR membrane or in reconstituted systems has been determined using saturation transfer EPR.[66,175,224-228] or

optical spectroscopic techniques.[209,229-232] A study of the effect of changes in the lipid environment or in temperature on protein mobility could provide clues as to how lipid-protein interactions modulate enzyme function.

Thus, in an effort to determine what is the physical basis of the break at 20°C in the ATPase activity, the rotational mobility of the protein has been measured as a function of temperature. It was initially reported that Arrhenius plots of the enzyme-rotational mobility in SR vesicles or in systems reconstituted with fluid lipids displayed a break at 20°C,[209,224,225] using either saturation transfer EPR or optical techniques to measure rotational mobility.

However, recent measurements under better experimental conditions[233] so as to make it possible to accurately measure the overall rotational mobility of the protein as a function of temperature, have shown linear Arrhenius plots of rotational mobility, with no breaks in the 4 to 37°C range.[210,234] These observations suggest that there is no abrupt temperature-dependent change in the shape or size of the rotating unit. Thus, these results make unlikely models in which the enzyme, in the absence of ATP, would undergo changes either in protein-protein associations or experience large-scale conformational changes at any given temperature.[209,225-227] Furthermore, the aggregation state of the ATPase does not change with temperature, as determined by freeze-fracture studies. Thus, if changes in lipid and protein occur at 20°C, they are not detected as changes in lipid fluidity or protein rotational mobility without ATP. Likewise, the translational diffusion of the enzyme does not change at 20°C.[236] Changes in the chain mobility of the boundary lipids at 20°C, which might be difficult to detect experimentally,[49] are unlikely to take place since such changes would be expected to affect the rotational mobility of the enzyme. An alternative possibility is that the ATPase undergoes a local conformational change at 20°, that might not be reflected in a change in overall protein-rotational mobility. In fact, several reports have described changes in the temperature dependence of several enzyme properties at 20°C,[202,207,208,237] although it is not clear yet whether these changes are related to internal motions relevant to the enzyme reaction cycle. Furthermore, no major conformational changes in the 10 to 34°C have been detected by infrared spectroscopy.[238]

However, it is important to point out that in the studies of Bigelow et al.[210] the rotational mobility was measured as a function of temperature for the enzyme in the absence of ATP. It is conceivable that changes in rotational mobility might take place during the reaction cycle, originating the 20°C breaks in Arrhenius plots of ATPase activity. It is interesting to note in this regard that recent experiments suggest changes in association-dissociation of ATPase monomers during the reaction cycle, as determined by the effect of vanadate and cross-linking agents on protein rotational mobility.[239,240] These observations clearly point out the need to carry out physical studies on different states of the ATPase during the reaction cycle, since this information might provide some explanation for the physical basis underlying the 20°C break in ATPase activity.

An important observation on the work of Bigelow et al. and Squier et al.[210,218] is that the apparent activation energy for ATPase activity above 20°C (11.8 kcal/mol) is very similar to the apparent activation energy of protein rotational mobility (11.2 kcal/mol), and lipid fluidity (11.3 to 11.8 kcal/mol), measured in the temperature range of 0 to 37°C. These results suggest that above 20°C protein rotational mobility determines the overall rate of the ATPase reaction. As discussed above (Section V.C), it is likely that the conformational changes associated with the ATPase reaction require a certain degree of protein-rotational mobility, which in turn is modulated by the fluidity of the lipid phase. The native SR lipids present above 20°C a fluid enough environment around the enzyme to allow the required rotational mobility of the protein. Either

substantial delipidation[200] or lipids in the gel phase[175,229] would prevent this rotational mobility and inhibit the ATPase activity.

Although other studies[178] do not agree with this interpretation, they are subject to experimental limitations[228] that might hinder the validity of the results.

E. Role of Lipids in Calcium Transport

While it seems that ATPase activity can take place regardless of the polar head of the phospholipid associated with the enzyme, provided a suitable bilayer thickness and fluidity are maintained, calcium transport seems to have more specific lipid requirements. Thus, it has been reported that in order to reconstitute the enzyme in sealed vesicles with a good coupling ratio of calcium transported to ATP hydrolyzed, phosphatidylethanolamine[241-246] or other lipids capable of adopting non-bilayer configurations are required.[244] However, other workers have reconstituted the enzyme only with phosphatidylcholine,[172,247] and have reported calcium transport with coupling ratios as high as 1.6 calcium ions transported per ATP hydrolyzed.[247]

In agreement with the studies describing a need for phosphatidylethanolamine for successful reconstitution of calcium transport, it has been reported that modification of the polar head group of phosphatidylethanlamine with fluorescamine in native SR vesicles uncouples calcium transport from ATP hydrolysis, without making the vesicles leaky.[59] However, phosphatidylethanolamine would not be specifically required but other cone-shaped lipids would allow efficient coupling when mixed in adequate proportions with bilayer-forming lipids.[244]

These results have been interpreted in terms of a requirement for lipids capable of adopting nonbilayer structures to efficiently couple calcium transport to ATP hydrolysis. However, it remains to be established why these particular lipids are needed in coupling.

It is conceivable that phosphatidylethanolamine might help in sealing the ATPase in the membrane,[243] since it is a cone-shaped lipid.[54] Furthermore, phosphatidylethanolamine extracted from the SR membranes undergoes a bilayer-to-hexagonal phase transition at −10°C (Figure 2). However, no such transitions are observed in the extracted SR lipids or in the native SR membranes (Figure 2). Nevertheless, it is conceivable that a small fraction of phosphatidylethanolamine, which is highly enriched in the outer monolayer, might adopt nonbilayer configurations during the Ca transport reaction, thus allowing efficient coupling.

On the other hand, it has been suggested that following enzyme phosphorylation by Pi a small amount of SR lipids becomes occluded,[248] but there is no information as to whether this occlusion involves a specific phospholipid such as phosphatidylethanolamine. Furthermore, addition of cholesterol to ATPase reconstituted with unsaturated phosphatidylethanolamine/phosphatidylcholine enhances calcium transport and increases coupling efficiencies.[246] It has been proposed that this enhancement is related to the bilayer-destabilizing effect of cholesterol, as revealed by ^{31}P NMR studies, without necessarily requiring actual formation of nonbilayer structures. Clearly, more structural information is needed in order to ascertain whether phosphatidylethanolamine or other cone-shaped lipids have a role in allowing efficient coupling.

Furthermore, a recent report disclaims any requirement for phosphatidylethanolamine in reconstitution of calcium transport,[249] and proposes instead that the 53,000-dalton protein present in native SR[250,251] is needed for successful reconstitution. Thus, the role of phosphatidylethanolamine in calcium transport is not definitely established yet, and additional studies are needed to ascertain this point.

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