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Structure, function and biosynthesis of sarcoplasmic reticulum proteins

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With a membrane that contains only a few proteins it is possible to ask how these proteins contribute to the structure and junction of the membrane and how the proteins are assembled in vivo *into a functional membrane.*

The sarcoplasmic reticulum is a membrane system unique to muscle tissue. In skeletal muscle, where it surrounds each myofibril, the membrane can occupy a very large percentage of the total cell volume. It plays an important role in muscle contraction by regulating intracelular Ca^{2+} concentrations. When the membrane system is fragmented it reforms vesicles with the same orientation *in vitro* as *in vivo*. Because these vesicles are so specialized for Ca^{2+} transport and because they are available in large quantities they have provided an excellent system for investigation into the mechanism of Ca^{2+} transport [1,2]. Investigations in this area have been advanced by the isolation and characterization of the proteins of the membrane, and these advances have permitted the development of other areas of investigation such as the biosynthesis of the membrane.

Protein composition

The sarcoplasmic reticulum contains only a few proteins. The ATPase, mol. wt 100,000, constitutes about two-thirds of the total protein of the membrane but perhaps as much as 90% of the intrinsic membrane protein [1]. The enzyme can be isolated in its active form containing phospholipid and small amounts of low molecular weight proteolipid which has a hydrophilic amino acid composition but contains covalently bound fatty acid. This enzyme, when incorporated into liposomes containing internal oxalate or phosphate, can catalyse ATP-dependent Ca^{2+} transport. Phospholipid is a co-factor since about 30 molecules of lecithin form an annulus around the ATPase, activating ATPase activity but probably not participating directly in movement of Ca^{2+} across the membrane. This Ca^{2+} translocating or ionophoric activity, must reside in the ATPase molecule or in the proteolipid. Since proteolipid fractionates with lipid rather than with the ATPase, the two proteins probably do not form a tight complex as do subunits of the complexes of the mitochondrial inner membrane. The proteolipid might interact functionally with the ATPase since the addition of optimal amounts of the proteolipid to reconstituted systems increases the efficiency of Ca^{2+} transport. Although it was proposed that the proteolipid might act as an ionophore in conjunction with the ATPase, the proteolipid does not show ionophoric activity when assayed in planar bilayers. Thus the role of the proteolipid in ion transport is not very well defined.

A group of at least four intrinsic membrane proteins of mol. wts 28,000-34,000 have been observed in sarcoplasmic reticulum membranes. Since these proteins have not been purified individually and characterized to any extent, little is known about them.

There are two extrinsic proteins associated with the inside of the sarcoplasmic reticulum – calsequestrin and the high-affinity calcium-binding protein. Calsequestrin (mol. wt 45,000) is very acidic and is able to bind some 45 mol of Ca^{2+} per mol of protein with a dissociation constant, in physiological ionic strength, of about 500 μM. The high-affinity Ca^{2+}-binding protein (mol. wt 55,000) is not so acidic as calsequestrin and binds correspondingly less Ca^{2+}. However, the protein binds 1 mol of Ca^{2+} per mol with a dissociation constant of 3–4 μM. Calsequestrin is a glycoprotein containing the 'core' sugars, 2 glucosamine and 3 mannose residues per molecule of protein: the high-affinity Ca^{2+}-binding protein contains no carbohydrate.

Structure and function of proteins

In order to understand the function of each of these proteins, their structure and localization must also be known. Studies with membranes reformed from the purified ATPase showed that 80–90 Å intramembrane particles and 40 x 60 Å projecting surface particles are both structural features of the ATPase protein [1]. Although the surface and intramembrane particles are randomly oriented in reconstituted vesicles, they are both associated almost exclusively with the cytoplasmic surface or leaflet in intact vesicles [1,3]. These studies lead to the conclusion that the ATPase is a transmembrane protein, asymmetrically arranged with a large portion of the protein extending into the cytoplasm but with very little protein extending into the lumen. Ca^{2+} and ATP are probably bound on the cytoplasmic surface where ATP is hydrolysed inducing translocation of Ca^{2+} through a transmembrane segment of the protein.

Calsequestrin, possibly as a Ca^{2+}–calsequestrin complex, makes up a fibrous matrix in some of the vesicular fragments of sarcoplasmic reticulum [4]. The calsequestrin-containing vesicles appear to be derived from the terminal cistemae of the sarcoplasmic reticulum since this is the only area where a matrix is observed *in situ*. Moreover, immunofluorescent labelling of rat skeletal muscle shows that staining for calsequestrin is limited to the area of the A-I junction, the region of cistemal abutment to the transverse tubular system [5]. By contrast, immunofluorescent staining for the ATPase is found throughout the sarcomere. Combined functional and structural data suggest that calsequestrin is the major site of calcium sequestration within terminal cisternae of the sarcoplasmic reticulum.

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Neither the exact localization nor the function of the other proteins is known with any certainty. Most, but not all, studies of localization of the high-affinity Ca^{2+}-binding protein have suggested that it is located in the lumen of the membrane, but not necessarily in the same region as calsequestrin. Its high-affinity binding site for calcium suggests that its function is regulated by Ca^{2+} fluxes. It has not been possible to assign any structural features to the proteolipid but it is almost certainly embedded in the bilayer. One or more of the intrinsic proteins of about 30,000 daltons have been tentatively assigned to structures in the junctional region of the sarcoplasmic reticulum and transverse tubular system [6] and proteins of 30,000 daltons are found in sarcoplasmic reticulum vesicles derived from this junctional region [7]. These proteins are exposed on the cytoplasmic surface of the vesicles.

Enzymatic reactions

The outline of the mechanism of Ca^{2+} transport has emerged from kinetic studies of the overall and partial reactions ofATP-dependent Ca^{2+} transport in intact vesicles and in the isolated enzyme. These studies have been reviewed in detail [1,2]. In summary, Mg-ATP, and Ca^{2+} bind to the cytoplasmic surface of the ATPase enzyme in the ratio of 1:2. ATP is hydrolysed with release of ADP on the outside, formation of an E-P complex and simultaneous translocation of Ca^{2+} to the luminal surface. Whereas Ca^{2+} is essential for E·P formation, Mg^{2+} is required for its decomposition; Pi is released on the cytoplasmic surface.

ATP-dependent Ca^{2+} transport is reversible: a gradient of Ca^{2+} across the membrane can drive the formation of ATP from E·P and ADP. However, ATP can also be synthesized by the isolated enzyme in the absence of a gradient [8]. If the enzyme is phosphorylated by Pi in the presence of Mg^{2+} and the absence of Ca^{2+} the phosphoryl group can be transferred to ADP when ADP and Ca^{2+} are added simultaneously to the phosphorylated enzyme. The possibility that conformational changes are induced in the enzyme by binding of the two divalent metals and that these conformational changes are responsible for Ca^{2+} movement has been discussed by Packer [9].

Active sites within the ATPase protein

Since Ca^{2+} is probably transported across the membrane through the ATPase itself regulated carriers or channels should be present in the protein and might well exist as entities separate from the site of ATP hydrolysis. The site of ATP hydrolysis has been measured by the location of the acyl phosphate transferred to the protein during ATP hydrolysis. The ionophoric site has been somewhat more difficult to measure. The ATPase increases permeability in liposomes but the induced permeability is non-specific, increasing for anions as well as cations [10]. When the ATPase was incorporated into the planar bilayer it was found to increase conductance of Ba^{2+}, Sr^{2+}, Ca^{2+}, Mg^{2+} and Mn^{2+}, but not of Zn^{2+} or monovalent metals and there was selectivity for cations over anions. Since the sarcoplasmic reticulum membranes transport only Ca^{2+} and Sr^{2+}, the two metals which activate ATP hydrolysis, this ionophoric site could not be considered to operate independently but must be considered to be under the direct control of the site of ATP hydrolysis which might permit selective entry of cations to the ionophoric site. The sites of Ca^{2+} binding which activate ATP hydrolysis are not necessarily the same structures involved in the ionophoric activity: Ca^{2+} may move from one site to another in its passage across the membrane.

Studies with CH_3HgCl and $HgCl_2$ suggest that sites of ATP hydrolysis and ionophoric activity do act in sequence [11]. Both inhibited ATPase and Ca^{2+} transport, but only $HgCl_2$ inhibited ionophoric activity. Dose-response curves showed that $HgCl_2$ inhibited Ca^{2+} transport at a lower concentration than that required to inhibit ATP hydrolysis as though its first target were the ionophoric site and its second target the ATPase. By contrast, CH_3HgCl inhibited the two activities concomitantly indicating that its only site of action was ATP hydrolysis. Ca^{2+} transport has also been uncoupled from ATP hydrolysis by other methods such as acid treatment and tryptic digestion.

Trypsin applied to sarcoplasmic reticulum vesicles cleaves the enzyme first into two fragments of mol. wts 45,000 and 55,000 and then cleaves the 55,000 dalton fragment into fragments of about 20,000 and 30,000 daltons. The first cleavage does not affect either ATP hydrolysis or Ca^{2+} transport but there is a loss of Ca^{2+} transport activity concurrent with the second cleavage of the 55,000 dalton fragment. The fragments have all been isolated and partially characterized. They can be aligned as N-Ac-met-20,000–30,000–45,000-COOH [12], and they probably all contain transmembrane segments [13]. The sequence of the first 25 amino acids comprising the N terminus of the 45,000 dalton fragment contains about 80% non-polar amino acid residues. This sequence would appear to represent a transmembrane sequence implying that the cleavage occurs at the point where the transmembrane sequence emerges from the bilayer.

Ionophoric activity was found in the 55,000 and 20,000 dalton fragments [14] while the site of ATP hydrolysis was located in the 55,000 and 30,000 dalton fragments [15]. Thus the two sites are separated in the primary sequence. Attempts have been made to isolate the two large fragments and to demonstrate their separate roles in Ca^{2+} transport. While it has been possible to achieve reconstruction of transport after dissociation of the two large fragments in SDS, it has not been possible to achieve dissociation, separation and reconstruction. Further cleavage of the 20,000 dalton fragment with CNBr releases two large fragments of 12,000 and 8,000 daltons [12]. The 12,000 dalton fragment is the N-tenninal while the 8,000 dalton fragment is the C-terminal portion of the 20,000 dalton fragment. It is not clear as yet whether one or two small peptide intervene between these two larger fragments. The 12,000 dalton, N-terminal fragment appears to contain the ionophoric site. The sequence of the first 30 amino acids in this fragment of about 110 residues is known, and this sequence does not have the hydrophobic character that is readily identified with a transmembrane segment [16]. Therefore, it is probable that the ionophoric site is contained in the unknown sequence between residues 30 and 110 of the molecule.

A randomly dispersed K^+, Na^+ channel has been demonstrated in sarcoplasmic reticulum vesicles [17] and a voltage-dependent K^+ channel has been observed when vesicles are fused to planar bilayers [18]. These channels may allow K^+ movements to counter C^{2+} movements during Ca^{2+} uptake and release.

What form might these ionophores take? From first principles it would appear that an ionophore would either carry ions across a bilayer in a cyclical fashion or create a channel across the bilayer. Whil it seems unlikely that a portion of the ATPase molecule could be mobile within a bilayer, permitting it to act as a carrier, channel formation seems very likely. Several transmembrane sequences appear

to exist in the ATPase and one of these is probably the sequence at the N terminus of the 45,000 dalton fragment. Analysis of channel-forming properties of transmembrane peptides has shown that as few as 17 amino acids can create an α helix that will span a membrane [19]. The core of an α helix is narrow and would be unlikely to permit passage of ions. Thirty-four amino acids would be sufficient to create a β helix across a membrane and the channel formed in this way could accommodate ions.

Biosynthesis

Development of sarcoplasmic reticulum in avian and mammalian muscle begins in the embryo but continues postnatally. Microsomes isolated from embryonic and neonatal muscle tissue change from multifunctional membranes, characteristic of endoplasmic reticulum, to highly specialized Ca^{2+}-transporting membranes having a high content of sarcoplasmic reticulum proteins, an increased density of 90 Å intramembrane particles and surface projections and an increased protein:lipid ratio.

The process of sarcoplasmic reticulum synthesis and assembly can be studied more readily in tissue culture since muscle differentiation is both synchronous and rapid. Muscle cells in culture go through distinct stages in their differentiation. During the first two days they divide and align. After about 50 h the aligned cells begin to fuse, and this process continues for the next two days. By the fifth day the cells are rather highly differentiated and some of the muscle straps display rhythmic contractions indicative of a well developed, internal contractile system. In order to follow development of the sarcoplasmic reticulum membrane, developing cells have been sampled at given time intervals for such indicators of synthesis as rate of incorporation of radioactive amino acids into immunoprecipitable proteins, intracellular development of the capacity to bind immunofluorescent antibodies against sarcoplasmic reticulum proteins, development of capacity for Ca^{2+}-dependent formation of phosphoprotein or the appearance of intracellular structures identifiable as the sarcoplasmic reticulum.

Present studies have been confined to the ATPase and calsequestrin, two v proteins which differ in character and in localization but which ultimately become essential components of the same membrane. The ATPase did not appear to be synthesized to any significant extent during the first 50 h of culture but the rate of synthesis increased dramatically after fusion began. ATPase synthesis was not dependent upon cell fusion since it was formed in cells blocked for fusion by growth in low Ca^{2+} and, moreover, in normal growth medium, the ATPase was detectable by immunofluorescence in mononucleated as well as multinucleated cells.

Calsequestrin synthesis was initiated well before initiation of ATPase synthesis and well before cell fusion began. Blockade of fusion by low Ca^{2+} at a time when calsequestrin synthetic rates were 40% of maximal, sharply diminished the synthetic rate. However, calsequestrin synthetic rates were unchanged from the normal pattern when the switch to low Ca^{2+} was made at 22 h, a time prior to initiation of calsequestrin synthesis.

These observations, indicating a lack of correlation between ATPase and calsequestrin synthesis, have been confirmed by immunofluorescence studies. The ATPase was absent from cells during the first 50 h of growth, but thereafter it could be found in granular foci throughout the cytoplasm in all fused and some unfused cells. The staining pattern in myotubes appeared longitudinally striped. By contrast, calsequestrin staining was found in some mononucleated myoblasts well before fusion had begun and well before staining of the ATPase was apparent. Instead of being present throughout the cell, however, calsequestrin was localized in a discrete, perinuclear region at the earliest time of appearance. This may represent the Golgi region or rough endoplasmic reticulum abutting the Golgi region. As development progressed, calsequestrin spread from the sharply defined perinuclear region into the cytoplasm, gradually spreading into the poles of the cells. At late stages, calsequestrin, like the ATPase, was found throughout the differentiated myotubes.

Morphological studies of differentiating muscle cells in culture showed smooth membranes budding from rough endoplasmic reticulum, implying that there are direct continuities between the lumens of rough endoplasmic reticulum and sarcoplasmic reticulum.

These observations, when considered in the light of what is known of membrane differentiation, have been rationalized as representing the following sequence of events [20]. The ATPase may be synthesized on membrane-bound polyribosomes, penetrating into, but not passing through, the membrane. Newly synthesized phospholipid in bilayer form may bind to the endoplasmic reticulum expanding its mass. Lateral segregation of the newly synthesized ATPase into the bilayer could create a growing point between rough endoplasmic reticulum and smooth sarcoplasmic reticulum. Since the ATPase contributes up to 90% of intrinsic membrane protein of the sarcoplasmic reticulum, incorporation of this protein into phospholipid would be synonymous with synthesis of the structure recognized as sarcoplasmic reticulum.

Calsequestrin may also be synthesized on membrane-bound polyribosomes but be secreted into the lumen of the rough endoplasmic reticulum where it would be glycosylated. It may then move into cisternal regions of the rough endoplasmic reticulum or into the Golgi region. Its subsequent movement from cisternal regions or from the Golgi region into specific areas of the lumen of the newly developing sarcoplasmic reticulum could result from reverse flow through luminal spaces from the endoplasmic reticulum into the sarcoplasmic reticulum or it could be packaged in the Golgi region and vesicles containing calsequestrin could then move into the cytoplasm, fuse with the developing sarcoplasmic reticulum and empty their content of calsequestrin into the sarcoplasmic reticulum lumen.

In vitro studies of the ability of free and bound polyribosomes to synthesize the ATPase and calsequestrin have proven that both proteins are formed almost exclusively on membrane-bound polyribosomes [21]. Recently it has been suggested that proteins formed on membrane-bound polyribosomes might have a signal sequence of hydrophobic amino acids that bind the nascent chain to the membrane but which are removed in the lumen of the membrane by processing enzymes [22,23]. Glycosylation might then occur in the lumen of the rough endoplasmic reticulum and Golgi. At present no evidence has been obtained for a signal sequence in the mammalian ATPase. The N-terminal amino acid of the ATPase is acetyl methionine which may be the product of the initiator codon. The first 30 amino acids of the ATPase are not especially hydrophobic [16]. This raises the question whether these amino acids do, in fact, react with the membrane and whether the N-terminal methionine is indeed located on the luminal side of the membrane. These questions will be answered as more detail becomes available on synthesis of this protein.

Calsequestrin almost certainly undergoes processing. One problem that obscures a simple analysis of processing is that two genetically predetermined forms of calsequestrin are known to exist in normal animals [24]. The possibility that these different-sized calsequestrins, isolated from muscle, represent forms plus and minus signal sequence has been ruled out since both forms have the same rather hydrophilic N-terminal amino acid sequence beginning with glutamate.

While all of the questions that can be raised regarding assembly of this membrane system have not yet been answered (or even raised) the system appears to have a great deal of potential as a model for the understanding of mammalian membrane assembly.

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