J. Membrane Biol. 116, 1–8 (1990)

The Journal of
Membrane Biology

Topical Review

Coupling of Catalytic and Channel Function in the Ca^{2+} Transport ATPase

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Introduction

Active transport of Ca^{2+} is a phenomenon of general interest owing to the role of Ca^{2+} in regulation of several biological systems and a need for control of the Ca^{2+} concentration in membrane-bound cellular compartments (Carafoli, 1987). In fact, several cDNA clones have been recently obtained, allowing demonstration of homologous Ca^{2+} transport ATPases in plasma and intracellular membranes of a wide variety of cells (MacLennan et al., 1985; Gunteski-Hamblin, Greeb & Shull, 1988; Lytton & MacLennan, 1988; Verma et al., 1988).

Vesicular fragments of sarcoplasmic reticulum (SR) provide a favorable system for mechanistic studies of active transport (Hasselbach & Makinose, 1961; Ebashi & Lipmann, 1962), as the SR membrane contains a dense assembly of Ca^{2+}-ATPase molecules which constitute the Ca^{2+} pump. The Ca^{2+}-ATPase is composed of a single large protein of 110,000 Da, containing 1001 amino acid residues. A large portion of the protein protrudes from the cytosolic surface of the membrane, i.e., facing outward in isolated vesicles. The rest of the protein is embedded through the phospholipid bilayer with a small portion extending from the lumenal side of the membrane.

We are here reviewing recent information derived from kinetic measurements, chemical modification, site-directed mutagenesis and sequence analysis. The information suggests that the perturbation triggered by formation of phosphorylated enzyme intermediate in the cytosolic portion of the ATPase is transmitted to a transmembrane, channel-like structure where bound calcium is subjected to vectorial displacement. This is the basic mechanistic feature whereby catalytic and transport activities are coupled.

Key Words transport · calcium · ATPase · binding · phosphorylation · catalysis

The Catalytic and Transport Cycle

The operation of the Ca^{2+} pump can be described by a minimal number of partial reactions, as shown in Table 1.

These reactions have been characterized in detail (Inesi, Kurzmack & Lewis, 1988), including determination of the equilibrium constants shown above as well as the individual rate constants. This reaction scheme, based on experimental data, directly relates the phosphorylation-dephosphorylation cycle of the enzyme with changes of the binding constant for Ca^{2+} (Inesi et al., 1980; Pickert & Jencks, 1984; Inesi, 1985), thus providing an explicit mechanism as well as an account of transport energetics.

In broad terms, formation of phosphorylated enzyme intermediate and calcium site occupancy are mutually exclusive (Jencks, 1980; Hill & Eisenberg, 1981; Tanford, 1983). Thereby, enzyme phosphorylation by ATP in the presence of calcium (Yamamoto & Tonomura, 1968; Makinose, 1969) results in vectorial translocation of bound calcium (Inesi, Kurzmack & Verjovski-Almeida, 1978). Conversely, enzyme phosphorylation by P_i occurs only in the absence of Ca^{2+} (Masuda & deMeis, 1973), and this intermediate is destabilized (i.e., its phosphorylation potential is increased) by Ca^{2+} binding (Knowles & Racker, 1975; de Meis & Tume, 1977).

Calcium Binding and Translocation

To gain a better understanding of active transport, it is useful to consider more detailed features of calcium binding to the ATPase in the absence of ATP and translocation of bound calcium upon addition of ATP.

In the absence of ATP, calcium binding to the SR ATPase at equilibrium yields cooperative iso-

Table 1. Sequence of partial reactions comprising the catalytic and transport cycle of the sarcoplasmic reticulum ATPase

$E + 2Ca^{2+}_{out} \rightleftharpoons E\ Ca_2$	$(3 \times 10^{12}\ M^{-2})$
$E\ Ca_2 + ATP \rightleftharpoons ATP\ E\ Ca_2$	$(1 \times 10^{5}\ M^{-1})$
$ATP\ E\ Ca_2 \rightleftharpoons ADP\ E\text{-}P\ Ca_2$	(0.3)
$ADP\ E\text{-}P\ Ca_2 \rightleftharpoons E\text{-}P\ Ca_2 + ADP$	$(7 \times 10^{-4}\ M)$
$E\text{-}P\ Ca_2 \rightleftharpoons E\text{-}P + 2Ca^{2+}_{in}$	$(3 \times 10^{-6}\ M^2)$
$E\text{-}P \rightleftharpoons E\ P_i$	(1)
$E\ P_i \rightleftharpoons E + P_i$	$(1 \times 10^{-2}\ M)$

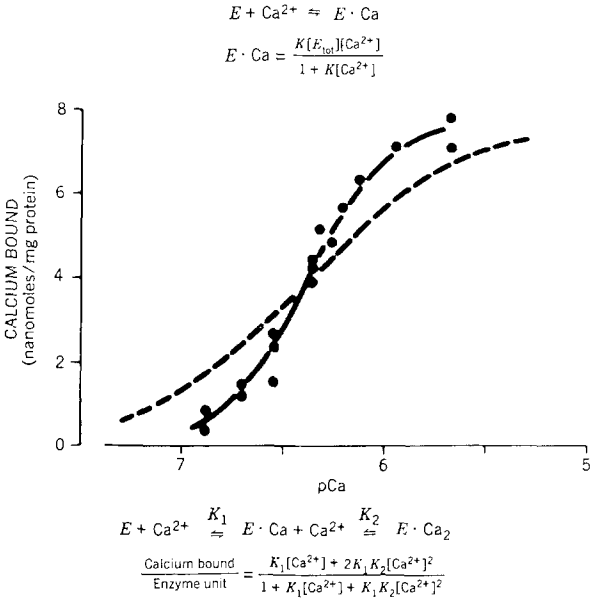


Fig. 1. High-affinity calcium binding to sarcoplasmic reticulum ATPase. The measurements were obtained at equilibrium and in the absence of ATP (Inesi et al., 1980). A good fit of the experimental points was obtained with a sequential binding model that explains the observed cooperativity (lower equation, solid curve). A simple, independent calcium-binding model does not yield a satisfactory fit (upper equation, dotted line). The maximal binding of 8 nmol/mg protein, corresponds to 2 mol of calcium per mol of enzyme, since the preparation used in these studies yields maximal levels of 4 nmol of phosphorylated enzyme intermediate per mg protein

therms that can be explained with sequential binding of two calcium ions per ATPase copy (Fig. 1). The sequential mechanism is further supported by isotope exchange experiments (Dupont, 1982; Inesi, 1987). It can be then demonstrated by rapid kinetic experiments that when ATP is added, the first Ca^{2+} acquired by the ATPase from the cytosolic side is also the first to be released onto the lumenal side (Inesi, 1987; Khananshvili & Jencks, 1988). These findings suggest that two calcium ions bind in single file within a protein crevice. This binding domain is sensitive to enzyme destabilization consequent to

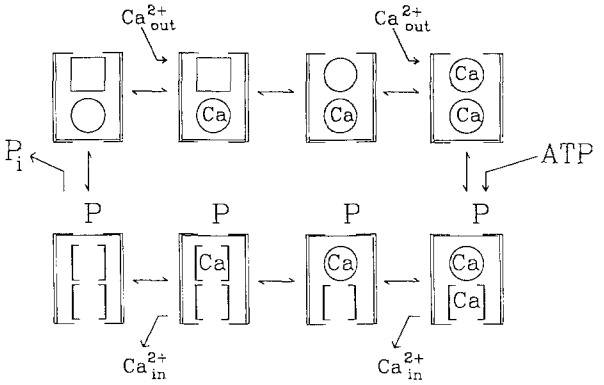


Fig. 2. Diagram of sequential translocation of bound calcium upon addition of ATP to sarcoplasmic reticulum ATPase. The sequential mechanism was derived from rapid kinetic measurements of single transport cycles

formation of the phosphorylated enzyme intermediate and provides a path for vectorial translocation of bound calcium (Fig. 2).

Structural Features of the Ca^{2+}-ATPase

The primary structure of the SR ATPase was elucidated partially by conventional amino acid sequencing (Allen, 1980), and then *in toto* by cDNA sequencing (MacLennan et al., 1985). As the cDNAs encoding a number of cation ATPases have been recently sequenced, it is of interest to compare the related amino acid sequences in search of homologies that may suggest common functional features (Table 2).

An important point of reference within the SR ATPase sequence is an aspartyl residue (Asp_{351}) which is phosphorylated when the enzyme intermediate is formed upon utilization of ATP (Bastide et al., 1973; Degani & Boyer, 1973). Therefore, this residue resides within the ATPase catalytic domain. Marked homology is noted in this region when various cation transport ATPases are aligned (Table 2). This homology establishes a common feature near the catalytic site of these cation transport ATPases, and identifies a segment that is undoubtedly involved in energy transduction.

Hydropathy plots of the SR ATPase sequence (MacLennan et al., 1985) reveal a large segment spanning from residue 316 to residue 759, of rather polar character, which obviously corresponds to the large portion of the ATPase molecule protruding from the membrane into the cytosol (Fig. 3). The hydropathy plots show also several segments of low polarity which are likely to acquire a helical conformation and to partition within the membrane bi-

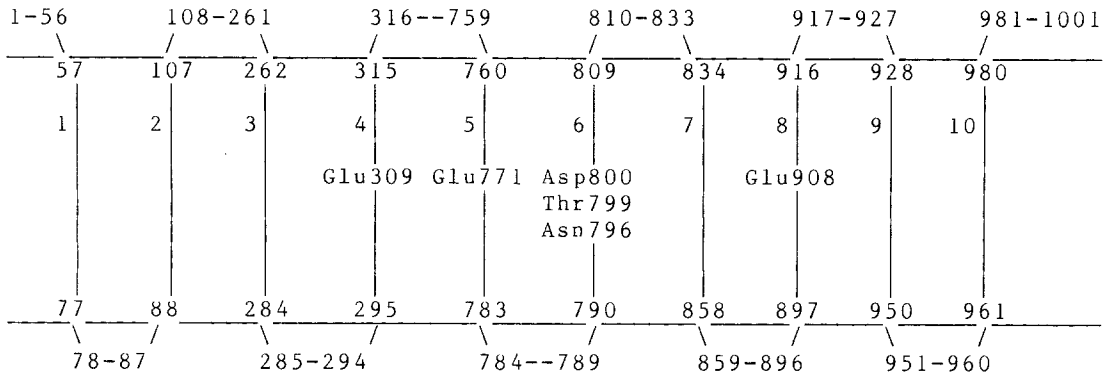


Fig. 3. Topology of the Ca^{2+}-ATPase in the sarcoplasmic reticulum membrane. Large segments at the top extend into the cytosol; short segments are exposed at the lumenal side of the membrane. The model shown is based on that of Clarke et al. (1989); the residues associated with calcium binding are shown within the transmembrane domain; helix numbers are shown beside each transmembrane segment. It should be noted that the available data are consistent with either 8 or 10 transmembrane segments

layer. MacLennan et al. (1985) suggested that 10 discrete transmembrane helices are formed by these segments, crossing the membrane inward and outward as five "hairpins," thereby allowing both amino and carboxyl terminal of the ATPase to remain on the cytosolic side. It should be pointed out that there is some uncertainty on the segment of each helix that is actually included between the membrane boundaries. The pertinent nonpolar segments are indicated by a line on the SR ATPase sequence (Table 2), and their partition within the membrane as recently proposed by Clarke et al. (1989; *see also* Green, 1989) is shown in Fig. 3.

In addition to the large cytosolic domain and the 10 transmembrane helices, other connecting segments are noted: the N-terminal 1–57, the fairly large 108–260 segment, the two small 810–830 and 917–928 segments, and the 987–1001 C-terminal are likely to be on the cytosolic side of the membrane. Finally, five short polar sequences constitute the connecting segments of the five transmembrane "hairpins" on the lumenal side of the membrane (Fig. 3).

As the amino acid sequence of a number of cation ATPases (derived from their cDNA) is now available, it is of interest to search for homologies that may suggest common functional domains. Extensive homology is noted (Table 2) in the Ca^{2+}-ATPases of fast and slow/cardiac muscle SR (MacLennan et al., 1985; Brandl et al., 1986). Lower homology is found when the Ca^{2+}-ATPase of SR is compared with the Ca^{2+}-ATPase of plasma membranes (Verma et al., 1988). This latter enzyme is larger owing to a 92-residue extension on the C-terminal (Table 2) that includes the calmodulin binding domain (not present in the SR ATPase).

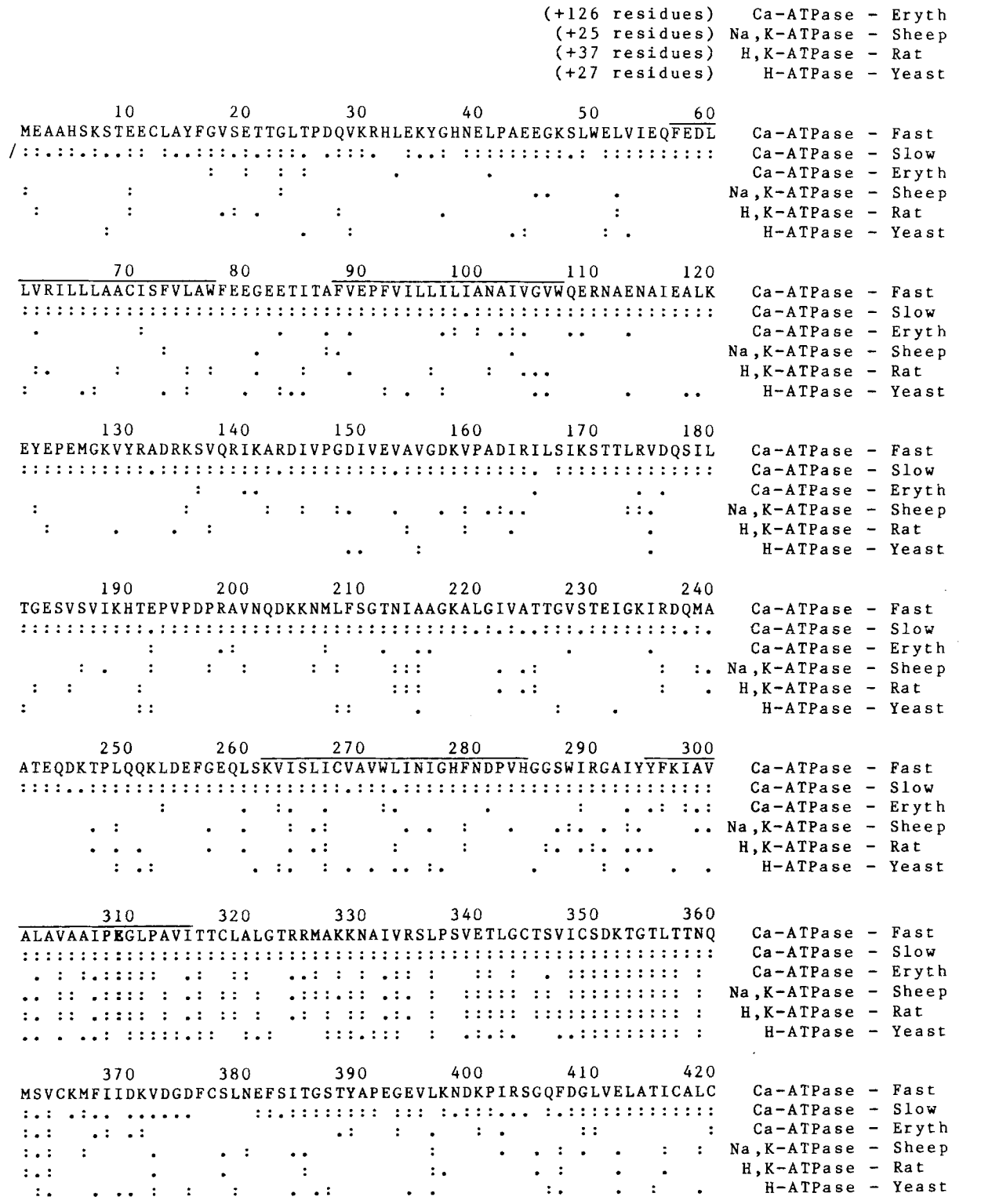
An even lower homology is noted when the Ca^{2+}-ATPase of SR is compared with other cation ATPases such as the sheep Na^+,K^+-ATPase (Shull et al., 1985), the rat gastric H^+,K^+-ATPase (Shull & Lingrel, 1986), and the yeast H^+-ATPase (Serrano, Kialland-Brandt & Fink, 1986) (Table 2). Nevertheless, all of these cation transport ATPases maintain a strong homology in the region around the phosphorylation site and the preceding transmembrane helix (residues 297–359 in Table 2). This persistent homology indicates that catalytic function and energy transduction are carried out through a similar mechanism in these cation-transport ATPases.

Catalysis and Calcium Binding Occur in Separate Domains

Location of the catalytic site in the cytosolic portion of the ATPase was established by the early identification of Asp_{351} as the residue undergoing phosphorylation upon ATP utilization (Bastide et al., 1973; Degani & Boyer, 1973). On the contrary, the location of the calcium-binding domain has remained elusive for a long time. It was first proposed, in this regard, that acidic residues in the "stalk" bridging the cytosolic and transmembrane domains of the ATPase, may be involved in calcium binding (MacLennan et al., 1985; Brandl et al., 1986). In fact, a corresponding tryptic fragment referred as to A2, was found to bind calcium with high affinity (Ludi & Hasselbach, 1984). Recent evidence, however, suggests that calcium binding occurs in the transmembrane domain of the ATPase.

It was originally observed by Dupont (1976) that calcium binding to the ATPase results in an increase of tryptophan fluorescence. More recently, analysis of the kinetics of tryptophan fluorescence

Table 2. Alignments with Ca^{2+}, Na^+/K^+, H^+/K^+ and H^+-ATPases



Comparison of the sequence of Ca^{2+}-ATPase of skeletal muscle with the sequences of other transport ATPases. The sequence shown is that of the fast Ca^{2+}-ATPase of sarcoplasmic reticulum. Identical (:) or related (.) residues in the other proteins are indicated. Blank spaces indicate unrelated amino acid residues in those positions. / = Start of sequence; \ = end of sequence; _____ = transmembrane segment (Clarke et al. (1989) model); where a sequence extends beyond that of the Ca^{2+}-ATPase the number of additional residues is shown in parentheses. The residues associated with calcium binding (*see* Fig. 3) are retained in the slow Ca-ATPase, only two of them are retained in the erythrocyte Ca-ATPase, and only one appears in the other ATPases.

Table 2. *Continued.*

```
       430       440       450       460       470       480
NDSSLDFNETKGVYEKVGEATETALTTLVEKMNVFNTEVRNLSKVERANACNSVIRQLMK   Ca-ATPase - Fast
::.::.::.::::::::::::::::: :::::::.::...:::.:::::::::::.::::   Ca-ATPase - Slow
    :     .   .   :  :  :: .     :  .    :   .            .    Ca-ATPase - Eryth
    .    .     :  :     .    :.         : .                .   Na,K-ATPase - Sheep
              :                  .                      : :    H,K-ATPase - Rat
     :    :          :  .           :        :         .  :     H-ATPase - Yeast

       490       500       510       520       530       540
KEFTLEFSRDRKSMSVYCSPAKSSRAAVGNKMFVKGAPEGVIDRCNYVRVGTTRVPMTGP   Ca-ATPase - Fast
::::::::::::::::.:.::.::... .:::::::::::::::::...:::.:.::::.   Ca-ATPase - Slow
:              :.    .              .   .        .:              Ca-ATPase - Eryth
         .:                .            .        ::      :     Na,K-ATPase - Sheep
     :   .: :  .                        : .                :   H,K-ATPase - Rat
. : :   : :                             .         :               H-ATPase - Yeast

       550       560       570       580       590       600
VKEKILSVIKEWGTGRDTLRCLALATRDTPPKREEMVLDDSSRFMEYETDLTFVGVVGML   Ca-ATPase - Fast
::.::.:::.:::.::::::::::::.:.: ::::: :.::..:..:::.:::: ::::   Ca-ATPase - Slow
                : .:           .    :   .         .      .      Ca-ATPase - Eryth
                        ..          : :   .       .   :    .   Na,K-ATPase - Sheep
             .       :             .::    .             . .    H,K-ATPase - Rat
 .   :      .              .     :                .   .  : :    H-ATPase - Yeast

       610       620       630       640       650       660
DPPRKEVMGSIQLCRDAGIRVIMITGDNKGTAIAICRRIGIFGENEEVADRAYTGREFDD   Ca-ATPase - Fast
:::: :: .:..:::.:::::::::::::::::::.::::::::::..:.:...:.:::::.   Ca-ATPase - Slow
      . :     :    .               .              ..       :   Ca-ATPase - Eryth
          :       .  :        . .       .     .. :       .     Na,K-ATPase - Sheep
      .        .     .    :.     :    :      .         .       H,K-ATPase - Rat
     .        . :   .    .   .   :    ..        : .              H-ATPase - Yeast

       670       680       690       700       710       720
LPLAEQREACRRACCFARVEPSHKSKIVEYLQSYDEITAMTGDGVNDAPALKKAEIGIAM   Ca-ATPase - Fast
:  ..::.:: .: ::::::::::::::.:::.:::::::::::::::::::::::::::   Ca-ATPase - Slow
        .       :                                  . ::          Ca-ATPase - Eryth
    :   :         .             . : .    .    :                 Na,K-ATPase - Sheep
.        .               :      :      .:               :      H,K-ATPase - Rat
      : :                     .     :      .                     H-ATPase - Yeast

       730       740       750       760       770       780
GSGTAVAKTASEMVLADDNFSTIVAAVEEGRAIYNNMKQFIRYLISSNVGEVVCIFLTAA   Ca-ATPase - Fast
::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::   Ca-ATPase - Slow
   .          .          .            : .   .       . .         Ca-ATPase - Eryth
  .                        .  .             :.  .  ..           Na,K-ATPase - Sheep
       ::     .                       .   .      :              H,K-ATPase - Rat
          :  .       . .              .:        .   .            H-ATPase - Yeast

       790       800       810       820       830       840
LGLPEALIPVQLLWVNLVTDGLPATALGFNPPDLDIMDRPPRSPKEPLISGWLFFRYMAI   Ca-ATPase - Fast
::.::::::::::::::::::::::::::::::::::::..:::.:::::::::::::.::   Ca-ATPase - Slow
        :       .                .  .        :   :         :   Ca-ATPase - Eryth
  .    : .    :  ... .                          .  .            Na,K-ATPase - Sheep
.   :    .          .                                   . .    H,K-ATPase - Rat
           .   .:         ..                   .           .     H-ATPase - Yeast

       850       860       870       880       890       900
GGYVGAATVGAAAWWFMYAEDGPGVTYHQLTHFMQCTEDHPHFEGLDCEIFEAPEPMTMA   Ca-ATPase - Fast
: :::::::::::::. :..:: :...::.::.::.::.:.:::.::.:::.: :::::   Ca-ATPase - Slow
                 . :            :            .             .   Ca-ATPase - Eryth
 :      :  :   :                     .        .          :     Na,K-ATPase - Sheep
. :  :    .       .        :          .      :                 H,K-ATPase - Rat
            :                 .               . : \              H-ATPase - Yeast
```

Table 2 continued on next page.

Table 2. *Continued.*

```
       910       920       930       940       950       960
LSVLVTIEMCNALNSLSENQSLMRMPPWVNIWLLGSICLSMSLHFLILYVDPLPMIFKLK    Ca-ATPase - Fast
:::::::::::::::::::::::.:::: ::::.::::::::::::::::.:::.::...    Ca-ATPase - Slow
       :        :             :  . .  :                   :     Ca-ATPase - Eryth
   .: .     .   .  .:.        : . :    .    ::         .       Na,K-ATPase - Sheep
    .          ::  :        . : ..  .:.   : .                   H,K-ATPase - Rat

       970       980       990      1001
ALDLTQWLMVLKISLPVIGLDEILKFIARNYLEDPEDERRK                       Ca-ATPase - Fast
.:..:::::::::::::: .::.:::.::::::  :\                           Ca-ATPase - Slow
            .    :     :  :  .             (+92 residues)       Ca-ATPase - Eryth
              :  .  .     : :      : :   \                   Na,K-ATPase - Sheep
   :      : : .        ..       .     \                         H,K-ATPase - Rat
```

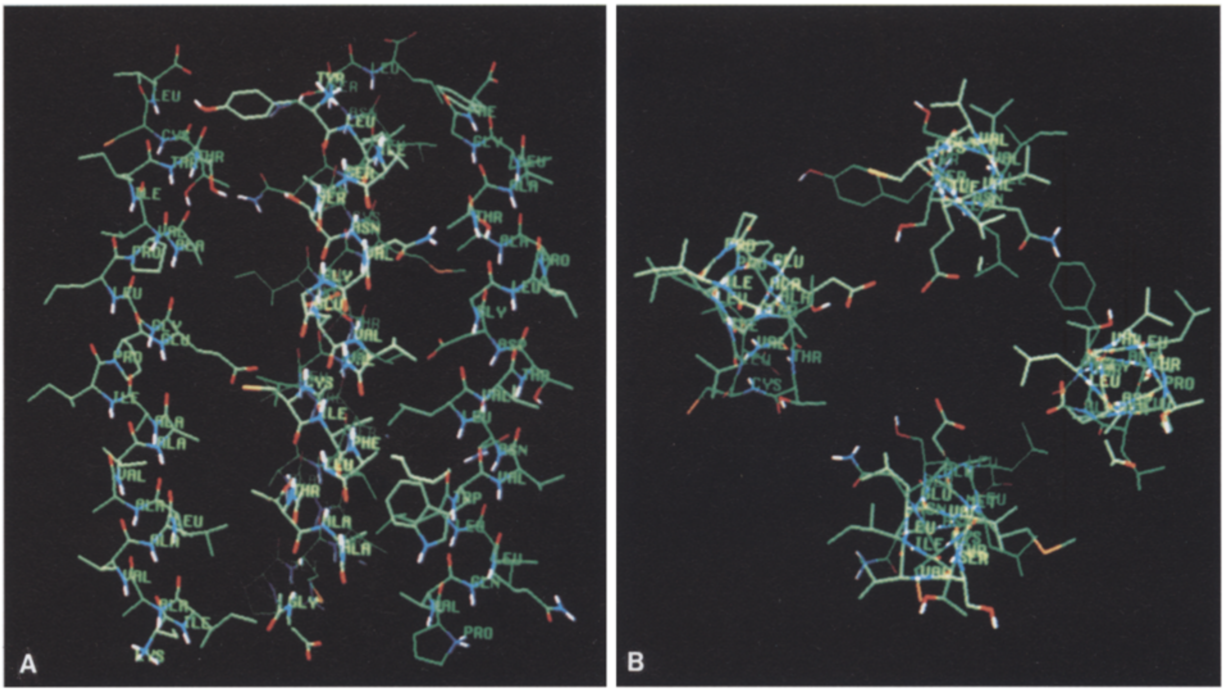


Fig. 4. Model of the four helices which contain Ca^{2+}-related residues. Helices 4–6 and 8 contain the six residues identified as critical to Ca^{2+} function in the Ca^{2+}-ATPase. These helices are shown in close contact with each other with a central opening through the structure. The side view (*A*) shows the close proximity of the four helices (helix 4, left; helix 5, center front; helix 6, right; helix 8, center back). The transverse view (*B*) shows the helices end on from the lumenal side of the membrane (helix 4, left; helix 5, top; helix 6, right; helix 8, bottom). The four acidic residues linked to calcium function can be seen projecting into the central cavity. The other two calcium-linked residues, although not visible in this view, are near by in helix 6

decay (Gryczinski et al., 1989) is consistent with multiple decay times whose Lorentzian distribution is clearly changed by calcium binding. It is noteworthy that 12 of the 13 tryptophan residues present in each ATPase copy are located near or within the transmembrane domain and are poorly accessible to acrylamide (as revealed by its fluorescence quenching effect). Since even the calcium effect is not influenced significantly by acrylamide, it is inferred that several or all the tryptophan residues within the transmembrane domain are affected by calcium binding. This is also confirmed by a rather long distance demonstrated by measurement of energy transfer between these tryptophans and fluorophores in the cytosolic portion of the ATPase. The calcium effect on the decay kinetics, as well as an observed increase in anisotropy, are attributed to stabilization of clustered transmembrane helices re-

sulting from calcium binding (Gryczinski et al., 1989).

Identification of amino acid residues which, within the transmembrane domain, are involved in calcium binding and/or its functional effects, was recently obtained by experimentation with mutated ATPase. Following the successful cloning of the cDNA encoding the SR ATPase (MacLennan et al., 1985), it has become possible to express the enzyme in cell culture systems (Maruyama & MacLennan, 1988; Karin, Kaprielian & Fambrough, 1989). Although calcium binding cannot be measured directly as yet, owing to the limiting amount of expressed ATPase, it is possible to measure the functional effect of calcium binding by autoradiographic detection of enzyme phosphorylation by ATP (which is calcium dependent). Extensive single-point mutation experiments have resulted in the identification of six residues which, when mutated singly, cause a loss of the enzyme to be phosphorylated by ATP in the presence of Ca^{2+} (Clarke et al., 1989). On the other hand, it was possible to demonstrate that these same mutations leave the phosphorylation domain intact since normal levels of phosphorylation can be obtained with P_i, a reaction that requires the enzyme to be free of calcium (Masuda & de Meis, 1973). In fact, addition of Ca^{2+} to the mutated ATPase does not inhibit phosphorylation by P_i. The six residues evidenced by the mutation experiments (Clarke et al., 1989) are Glu_{309}, Glu_{771}, Asn_{796}, Thr_{799}, Asp_{800} and Glu_{908}, all of which reside within the transmembrane helices (Fig. 3). The experiments with mutated ATPase indicate that these residues are closely involved in calcium-linked functions, although it is not possible to demonstrate which of them participate directly in calcium binding. Furthermore, it is not yet possible to establish whether these residues are involved in binding one or both of the calcium ions known to bind to each ATPase copy.

The specificity of the six residues listed above is underlined by other experiments in which several single-point mutations did not result in interference with calcium-binding functions (Clarke et al., 1989). A search for the six residues in the aligned sequences of other cation ATPases reveals that they are present in both fast and slow/cardiac muscle SR ATPases. Two of them (Glu_{309} and Glu_{908}) are also present in the plasma membrane Ca^{2+}-ATPase, only one (Glu_{309}) is present in the Na^+,K^+-ATPase and in the gastric H^+,K^+-ATPase, and none in the yeast H^+-ATPase (Table 2). On the other hand, it is possible to find acidic residues in neighboring segments that may participate in complexation with an analogous mechanism but different cation specificity (Green, 1989).

The Possible Role of an ATPase Channel in Translocation of Bound Calcium

Transmembrane helices are known to cluster and form water-filled pathways for passive fluxes of electrolytes in channel proteins (Noda et al., 1983; Noda et al., 1984; Grenningloh et al., 1987; Papazian et al., 1987; Schofield et al., 1987; Tanabe et al., 1987). The experimental findings reviewed above raise the possibility that the transmembrane helices of the ATPase may form a channel structure which is involved in active transport. The four helices containing the six residues related to calcium binding are of particular interest. Molecular graphic modeling of these four helices (4–6 and 8 in Fig. 3) reveals that they have an amphiphilic character, with polar and charged residues predominantly on one face of each helix, and with hydrophobic residues on the opposite face. These feature are consistent with their participation in a channel-like structure by clustering with their polar faces toward the interior of the channel and their hydrophobic faces toward the interior of the membrane bilayer.

There are several possible arrangements of helices 4–6 and 8, depending on how they relate to each other and to the other helical segments of the ATPase within the membrane bilayer. A possible arrangement of the four helices is represented in Fig. 4, showing the six amino acid residues related to calcium binding by the mutation experiments. It is apparent that at least five of these residues can face each other in a favorable arrangement for cation complexation. It is noteworthy that helix 4 and helix 5 are connected by the large cytosolic ATPase segment that comprises the catalytic and phosphorylation domain (Fig. 3).

Clustering of only four helices constitutes a quite narrow channel (Fig. 4*B*), with little room for participation of water in high order complexation. It is apparent that the six amino acid residues mentioned above can provide a sufficient number of oxygen atoms for direct cation complexation. It should be pointed out that while the water-filled pores of channel proteins allow passive fluxes of electrolytes at rates of the order of 10×10^8 sec^{-1}, the ATPase channel promotes active transport of Ca^{2+} at rates of the order of 20 sec^{-1}, and (in the absence of ATP) allows passive fluxes of Ca^{2+} at rates as low as 0.2–0.4 min^{-1} (Inesi & de Meis, 1989). This low permeability is related to calcium binding within the channel, providing a gating mechanism that favors the efficiency of the pump.

Conclusive Remarks

Functional and structural evidence indicates that two calcium ions bind sequentially in an ATPase

crevice. The transmembrane domain formed by clustered ATPase helices is involved in calcium binding and/or related functional effects. Although it is not clear whether the membrane domain involves binding of one or both calcium ions, it is certain the six amino acid residues within this domain are involved in activation of the catalytic site by Ca^{2+}, permitting utilization of ATP. In turn, the bound calcium is displaced into the lumen of the vesicle upon phosphorylation of the catalytic site by ATP. The distance between catalytic and calcium binding domain is quite large (i.e., from the cytosolic to the transmembrane portion of the enzyme), and the allosteric mechanism of transduction involves most likely a segment of the ATPase sequence that includes the phosphorylation site and the transmembrane helix number 4 (Fig. 3). This segment is highly conserved not only in the Ca^{2+}-ATPases, but also in the Na^+,K^+-ATPase, the H^+,K^+-ATPase and the H^+-ATPase (Table 2).

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Received 9 August 1989; revised 2 October 1989