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Sarcoplasmic reticulum calcium pump: A model for Ca^{2+} binding and Ca^{2+}-coupled phosphorylation

(Ca^{2+}-ATPase/active transport/transport stoichiometry/"hinge bending")

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ABSTRACT **The conventional alternating access model for Ca^{2+} transport by the sarcoplasmic reticulum Ca^{2+} pump is modified, partly on the basis of the proposed MacLennan–Green domain structure for the Ca^{2+}-pump protein. The present model divides the uptake state (E_1) of the protein into three substates, differing in the condition of the Ca^{2+}-binding domain. The domain is an open cavity in the first substate and can bind only a single Ca^{2+} ion. A fast "jaw-closing" (or "hinge-bending") step then partially closes the cavity to generate the second substate that has a second Ca^{2+}-binding site. Occupation of this site is followed by another jaw-closing step that closes the binding cavity and occludes the bound ions. The subsequent translocation step (to form E_2) remains unchanged from previous models. The modified model predicts a constant transport stoichiometry of two Ca^{2+} per pump reaction cycle. It suggests a plausible mechanism for coupling between Ca^{2+} binding and ATP utilization: the model predicts (in agreement with experiment) that Ca^{2+} binding should be a mandatory requirement for phosphorylation of the pump protein, though ATP binding *per se* does not require Ca^{2+}. The model is consistent with high cooperativity in equilibrium binding of Ca^{2+}, both in the absence and presence of ATP.**

The conventional model for the operation of the sarcoplasmic reticulum calcium pump is the alternating access model originally proposed by de Meis and Vianna (1). The model provides a simple and plausible mechanism for reversible free energy transfer between ATP and the transported Ca^{2+} ions, which involves the alternation of the pump protein between two conformational states, here called E_1 and E_2, in which the binding sites for Ca^{2+} are accessible from opposite sides of the membrane. The chemical potential of the transported ions (essentially equivalent to the binding affinity) alters in synchrony with the change in access, so that Ca^{2+} ions can be taken up at low concentration in one conformation (E_1) and discharged to a high concentration medium, normally the sarcoplasmic reticulum lumen, in the other conformation (E_2). There is a simultaneous change in phosphorylation potential between the phosphoenzyme derivatives ($E_1{\sim}P$ and $E_2{-}P$) to provide energetic balance. The rationale and energetics of the de Meis model have been elaborated and embellished in earlier papers from this laboratory (2–4).

It is established that two Ca^{2+} ions are normally transported in each cycle of the pump (1, 5). Moreover, direct binding studies show that E_1 has two high-affinity binding sites for Ca^{2+} and that binding to otherwise unliganded pump protein is a highly cooperative function of the Ca^{2+} concentration (6). The original de Meis model did not address the problem of Ca^{2+} uptake by these sites: it simply specified unliganded E_1 and Ca_2E_1 as sequential states of the protein. Earlier papers from our laboratory (7, 8) made the simple assumption that the two Ca^{2+}-binding sites of E_1 are essentially identical and equally accessible to Ca^{2+} from the adjacent aqueous solution. Binding cooperativity in our model was generated by an allosteric mechanism that depended on an unfavorable equilibrium between E_1 and E_2 in the unliganded state (7, 9). Hill and Inesi (10, 11) also postulated equally accessible sites and generated binding cooperativity by means of a formal (structurally undefined) interaction parameter that allowed binding at one site to affect binding affinity at the other sites: their model was actually a four-site model intended to apply to a dimer of the pump protein. Hill and Inesi considered pH dependence of Ca^{2+} binding in some detail; they ascribed it entirely to competition between H^+ and Ca^{2+} for the four binding sites in their model. This is probably unrealistic since the $E_1 \rightleftharpoons E_2$ equilibrium itself is known to be strongly pH-dependent (12, 13).

Several authors (14–17) have provided experimental evidence to indicate that the Ca^{2+}-binding sites in E_1 are in fact not identical or equally accessible. They suggest on the contrary that only one of the sites is accessible initially and that occupation of this site triggers a major (and *slow*) conformational change, which renders a second site available. This suggestion is again a formal one in the sense that the problem of how such "triggering" might be related to protein structure was not addressed. This model does, however, lead to yet another way to generate equilibrium-binding cooperativity by assigning a higher binding affinity to the induced site than to the first binding site (6, 16).

We present here a model for sequential Ca^{2+} binding to E_1 that incorporates the notion that there is a state with only one available binding site that must be occupied before a second binding site can be created. Our model is structurally quite specific and is based on the structural arrangement of functional domains for the Ca^{2+}-pump protein that has been proposed by MacLennan and coworkers (18, 19) on the basis of the amino acid sequence of the protein. It differs in an important way from the formal sequential models of the previous paragraph in that the conformational change between binding of the first and second Ca^{2+} ions is a minor (and *rapid*) change of the "hinge-bending" type known to occur in several other enzymes: it is a change between substates of E_1. The major transition in the reaction cycle remains the reversible $E_1 \rightleftharpoons E_2$ transition. The energetic basis for pump operation tied to that transition remains unaltered.

The justification for presenting this modified model here is not simply the need for a concrete hypothetical picture of how Ca^{2+} is bound. Without our intending it, the model provides surprisingly simple possible answers to several other questions of general interest. How is it possible to have a *fixed transport stoichiometry*—a quantization of the number of ions being translocated? How can one account for the "occlusion" of bound transport ions observed not only in the Ca^{2+}-pump cycle but also for Na^+/K^+ pumps (20–22)? Why is it that Ca^{2+} is not required for ATP binding, whereas two bound Ca^{2+} ions are mandatory before phosphorylation of the protein can occur (5)? These mechanistic features follow

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automatically from our attempt to do no more than design a sequential binding model on the basis of the MacLennan–Green domain structure.

DESCRIPTION OF THE MODEL

Ca^{2+}-Binding Domain. Our model is an extension of the conventional alternating access model (1–8) that retains the two principal conformational states: E_1, with high-affinity Ca^{2+}-binding sites for uptake of Ca^{2+} from the cytoplasm, and E_2, with low-affinity sites, from which Ca^{2+} is discharged into the sarcoplasmic reticulum lumen in the normal "forward" operation of the pump. The modification we have made consists of allowing the state E_1 to exist in three closely related substates, called E_x, E_y, and E_z, as shown in Fig. 1.

The particular substates proposed in Fig. 1 have a sound structural basis in that they are suggested directly by the amino acid sequence of the Ca^{2+}-pump protein recently published by MacLennan *et al.* (18) and the structural arrangement of functional domains of the protein based on that sequence (18, 19). In that arrangement, the Ca^{2+}-binding region of the protein is assigned to a stalk composed of several α-helices, located just outside the lipid bilayer domain of the membrane. The domain as a whole has a high density of negative charges, and this feature is a key element in our proposal. It suggests that the stalk helices may, in the absence of bound Ca^{2+}, be forced apart by electrostatic repulsion to create two subdomains with a cavity or cleft between them, as illustrated by conformation E_x of Fig. 1. Binding of the first Ca^{2+} might bring the subdomains together, thereby generating conformation E_y and concomitantly a second Ca^{2+}-binding site. The representation in Fig. 1 suggests that another small movement of the cavity walls to completely surround the second bound ion might then follow to generate conformation E_z.

The final step in Fig. 1 symbolizes the *major* conformational rearrangement that is involved in Ca^{2+}-ion translocation—i.e., formation of conformation E_2. Whether or not the transition to E_2 is physically possible in the absence of phosphorylation is a moot question that we need not address because uncoupled translocation must in any case be energetically impossible since the chemical potential of bound Ca^{2+} in state E_2 is much higher than it is in any of the substates of E_1. In practice, therefore, transition to state E_2 can occur only after the pump protein has been phosphorylated, so that the latent energy of the acyl phosphate link can provide the necessary thermodynamic driving force for raising the Ca^{2+} potential (1–4).

We shall comment further below on the linkage between Ca^{2+} binding and phosphorylation. The more important point in relation to Ca^{2+} binding *per se* is that the translocation step as here visualized cannot occur at all unless both Ca^{2+}-binding sites are occupied: the pump protein can make the transition to E_2 only after the binding cavity in E_1 is completely closed by formation of substate E_z. The model of Fig. 1 thus compels one to think in terms of a rigidly fixed transport stoichiometry. This is not intentional. We had no preconceived desire to generate a rigidly fixed stoichiometry model, but the structural considerations leading to Fig. 1 appear to make it inevitable. The model likewise inevitably leads to occlusion of the Ca^{2+} ions, as has been experimentally observed (20, 21). The bound ions in state Ca_2E_z are not directly accessible from either side of the membrane.

Fig. 1 raises the possibility that binding of Ca^{2+} to E_2 (or discharge in the normal mode of the pump) might involve similar successive subconformations to those we have postulated for E_1. There is no theoretical need for such a parallel, and the possibility is in any event not experimentally testable at present, because the Ca^{2+}-binding sites on E_2 have only very low binding affinity, an essential aspect of the pump mechanism (1–4). This means that the reversible association of Ca^{2+} with these sites can be studied only at millimolar levels of Ca^{2+}, conditions under which successive elementary steps will be too fast to be detected individually. In calculations based on the present model, we have treated Ca^{2+} binding to E_2 or E_2—P as a process at local equilibrium, which obviates the need for decision as to the exact pathway.

It should be noted that the $CaE_x \rightarrow CaE_y$ and the $Ca_2E_y \rightarrow Ca_2E_z$ transitions in Fig. 1 resemble the "hinge-closing" transition in hexokinase after glucose is bound (23). The latter is a fast transition with a rate constant on the order of 10^4 sec^{-1} or higher (24). There is no reason to expect the $E_x \rightarrow E_y$ and the $E_y \rightarrow E_z$ transitions for the Ca^{2+}-pump protein to be significantly slower, so in the absence of direct evidence to the contrary, we can expect these transitions to be relatively fast in comparison with the major alternating access transitions, $E_2 \rightarrow E_x$ and the reversal of that conformational change in the phosphorylated state, $E_z{\sim}P \rightarrow E_2$—$P$. A subsequent paper, discussing the relation between this model and experimental kinetic data, will show that a relatively large rate constant for the $CaE_x \rightarrow CaE_y$ transition is in fact essential if we are to avoid discrepancies with experimental data.

Linkage to Phosphorylation. The second part of the model involves coupling between Ca^{2+} binding and phosphorylation. Some sort of structural link between the Ca^{2+}- and ATP-binding sites is essential for a complete description of the pump mechanism, and the hypothesis that the linkage may be part of the cavity-closing transition of Fig. 1 is attractive.

It was shown some time ago by Mitchinson *et al.* (25) that the ATP-binding domain of the Ca^{2+}-pump protein is distinct from the structural domain that contains the phosphorylation site (i.e., the aspartic residue that becomes phosphorylated in the $E_1{\sim}P$ state of the pump protein). In the structural model proposed by Brandl *et al.* (19), there is a postulated "hinge" to move these two domains together (i.e., to move bound ATP to a position where it can transfer its terminal phosphate groups to the phosphorylation site). In Fig. 1 we also introduce a hinge at the bottom of the Ca^{2+}-binding cavity. It is an attractive hypothesis to suggest, as is done in Fig. 2, that these two proposed hinges may be one and the same—i.e.,

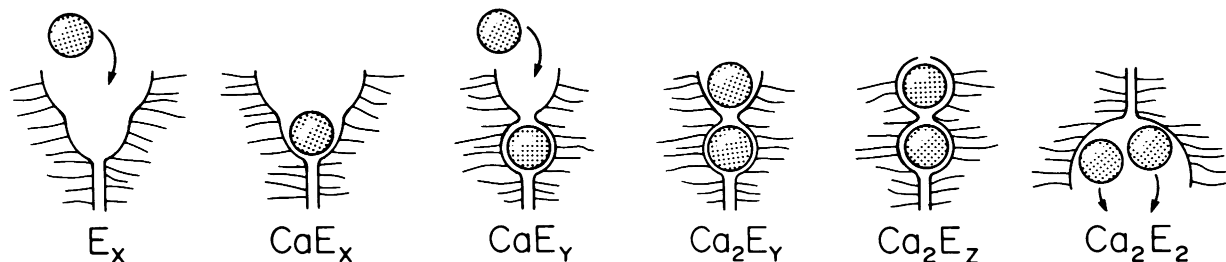

FIG. 1. Pictorial representation of the successive stages of the Ca^{2+}-binding domain of the protein. The binding cavity is assumed to extend physically into the cytoplasm, and its inner surface is assumed to possess a net negative charge.

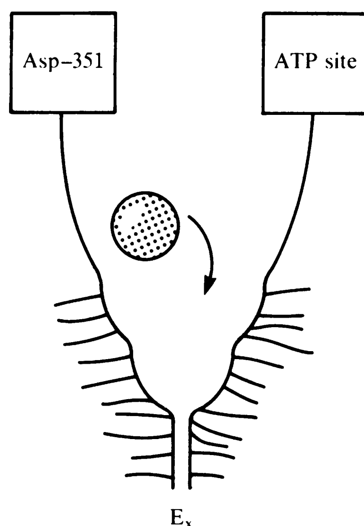

FIG. 2. Coupling between phosphorylation and Ca^{2+} binding. The ATP-binding site and the phosphorylation site at residue 351 are initially separate (18, 19). If these sites are rigidly linked to opposite sides of the Ca^{2+}-binding cavity, then they may move into close proximity as we pass from E_x to Ca_2E_z (see Fig. 1).

that the ATP-binding domain and phosphorylation domains may be connected to opposite sides of the Ca^{2+}-binding domain, so that the movement that closes the Ca^{2+}-binding cavity simultaneously imparts the movement required for phosphate-group transfer.

This suggestion would be consistent with a so-far unexplained experimental observation—namely, that the presence of two bound Ca^{2+} ions is required before phosphorylation can occur, whereas simple binding of ATP has no requirement for Ca^{2+} at all (5). There is no known energetic basis for a Ca^{2+} requirement for phosphorylation. One cannot here invoke the kind of *a priori* argument that we used above in discussing phosphorylation as a prerequisite for ion translocation.

EQUILIBRIUM BINDING OF Ca^{2+}

In equilibrium binding of a ligand to a protein with two binding sites, one can, in theory, measure two and *only two* equilibrium constants, no matter how complex the detailed model. A convenient pair of equilibrium constants for Ca^{2+} binding is defined as

$$K_{exp,1} = [ECa]/[E][Ca^{2+}] \quad [1]$$

and

$$K_{exp,2} = [ECa_2]/[ECa][Ca^{2+}], \quad [2]$$

where E represents all unliganded protein without making a distinction between different conformational states, and ECa and ECa_2 likewise represent liganded protein regardless of conformational state.

Binding of Ca^{2+} to the pump protein has been measured as an equilibrium process in the absence of ATP, ADP, or P_i, and it is known to be thermodynamically cooperative (6). The experimental binding curve is then usually characterized by its midpoint,

$$-\log[Ca]_{1/2} = \frac{1}{2}\log K_{exp,1} + \frac{1}{2}\log K_{exp,2}, \quad [3]$$

and by the degree of cooperativity. Two measures of cooperativity have been employed: the ratio of experimental association constants,

$$\rho = (K_{exp,2}/K_{exp,1}), \quad [4]$$

and the Hill coefficient,

$$n_H = d\log[\theta/(1-\theta)]/d\log[Ca^{2+}], \quad [5]$$

where θ is the fractional saturation of the total binding capacity (both sites). The Hill coefficient is itself somewhat dependent on θ, and it is customary to use the value at the midpoint ($\theta = 1/2$), which is directly related to ρ by the equation

$$n_H = 1 + (2\rho^{1/2} - 1)/(2\rho^{1/2} + 1). \quad [6]$$

We have used the results of Inesi *et al.* (6) near pH 7 as a basis for our analysis. Their data have $\log[Ca]_{1/2} \approx -6.5$ (molar units), $\rho = 420$, and $n_H = 1.82$. Their values of n_H and ρ are not self-consistent: the latter corresponds, by Eq. **6**, to $n_H = 1.95$. Self-consistent measures of cooperativity for their results are $\rho = 100$ and $n_H = 1.90$, which leads to $K_{exp,1} = 3 \times 10^5$ and $K_{exp,2} = 3 \times 10^7$ M^{-1} to obtain a binding midpoint in agreement with experimental results.

A formal reaction scheme corresponding to our model is shown in Fig. 3. The scheme formally defines five true equilibrium constants: two Ca^{2+}-binding constants, $K_{Ca,1}$ and $K_{Ca,2}$, and three conformational transition constants, K_0, K_3, and K_4, related to $K_{exp,1}$ and $K_{exp,2}$ by

$$K_{exp,1} = K_{Ca,1}(1 + K_3)/(1 + K_0) \quad [7]$$

and

$$K_{exp,2} = K_{Ca,2}(1 + K_4)K_3/(1 + K_3). \quad [8]$$

This means that there is no difficulty in fitting our model to the experimental values of $K_{exp,1}$ and $K_{exp,2}$. Many possible combinations of the model's K values will serve this purpose. For two of the constants, $K_{Ca,2}$ and K_4, assignment of independent values is not necessary at all. Only the product $K_{Ca,2}(1 + K_4)$—an "effective" association constant for the second Ca^{2+} ion—affects the results.

There are also limitations. $K_{Ca,2}(1 + K_4)$ must be kept below 10^8 M^{-1} because tighter binding would lead to an unacceptably slow rate for Ca^{2+} release, as will be shown in a later paper. With this limitation, Eq. **8** also sets a limit on K_3. There is also a limit on the value of K_0, which must be close to unity on the basis of experimental measures of the E_2/E_1 ratio in the absence of Ca^{2+} (12, 13). With these considerations in mind, we have set $K_0 = K_3 = 1$, which with Eqs. **7** and **8** leads to $K_{Ca,1} = 3 \times 10^5$ M^{-1} and $K_{Ca,2}(1 + K_4) = 6 \times 10^7$ M^{-1}. This is not a unique set of constants, but the choice of a different set within the prescribed limits would not affect any comparisons with experimental data that we have made so far.

Ca^{2+} Binding in the Presence of ATP. Kinetic response of pump activity to Ca^{2+} is also a cooperative function of $[Ca^{2+}]$, but the measurement is of course always in the presence of ATP, usually at millimolar levels, where ATP-binding sites in all relevant intermediate states are saturated or nearly so. It

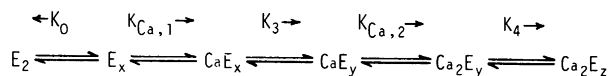

FIG. 3. Reaction sequence for reversible Ca^{2+} binding to the high-affinity sites of E_1 in the absence of ATP, ADP, or P_i. The first species in this scheme, E_2, is absent from Fig. 1 and is included here because it is well established that the unliganded Ca^{2+}-pump protein is partly or even predominantly in the E_2 state (7, 12, 13). Species Ca_2E_2 of Fig. 1 is omitted because it exists only in the phosphorylated form of the protein (see text). All steps of the scheme are reversible.

is therefore desirable that the model should show cooperative equilibrium binding of Ca^{2+} in the presence of ATP as well as in its absence (i.e., when the species E_2, E_x, etc. of Fig. 1 are replaced by the corresponding complexes with ATP).

To address this question, it is necessary to take into account the explanation we have previously offered (26) for the anomalous biphasic kinetics of ATP activation of the pump at constant $[Ca^{2+}]$. This involved binding of ATP to both the E_1 and E_2 conformations of the protein, with a much higher binding constant (K_{ATP}) for E_1, from which it could be predicted from the theory of linked functions (27) that ATP should accelerate the transition from E_2 to E_1. Anomalous kinetics of steady-state activation by ATP was quantitatively explained in this way, not only for the Ca^{2+} pump but also for Na^+/K^+ pumps (26).

This assignment of two different binding constants for ATP leads to a predicted effect of ATP on Ca^{2+}-binding equilibria even if we assume (as we have done) that there is no direct interaction between the binding sites for Ca^{2+} and ATP (i.e., $K_{Ca,1}$ and $K_{Ca,2}$ are unaffected by ATP binding). The reason is that the principle of linked functions (27) requires that a dependence of K_{ATP} on the protein conformational state must lead to a reciprocal dependence of the conformational transition equilibria on whether or not ATP is bound. Since these transition equilibria (K_0 and K_3) enter into the equations for $K_{exp,1}$ and $K_{exp,2}$, the equilibrium binding isotherms must also depend on whether or not ATP is bound.

The present mechanism for Ca^{2+} binding creates three substates of the E_1 conformation, necessitating more precise localization of the change in K_{ATP}. We have assumed that K_{ATP} changes with the major conformational transition between E_2 and E_x but that it is the same in all three substates of E_1. This means (Fig. 3) that K_0 is changed (*reduced* in value) by the presence of ATP and that the E_2 to E_x transition is accelerated, but K_3 and K_4 and $E_x \rightarrow E_y \rightarrow E_z$ are unaffected. Eqs. **7** and **8** show that changing K_0 from 1 to a smaller value will have only a trivial effect on Ca^{2+}-binding equilibrium and that the binding will therefore continue to be highly cooperative when ATP is bound.

We also have tested the alternative assumption that K_{ATP} has the same value in E_2 and E_x and increases between CaE_x and CaE_y, which means that K_3 instead of K_0 is altered by ATP binding. When this is done, it is not possible to maintain Ca^{2+}-binding cooperativity when ATP is bound, unless $K_{Ca,2}(1 + K_4) > 10^9$ M^{-1}, a possibility excluded for the reasons given above.

DISCUSSION

The model for Ca^{2+} binding presented in this paper is based on the recent structural work of MacLennan and coworkers (18, 19) and could not have been designed without it. It should be noted, however, that MacLennan and coworkers themselves have postulated a mechanism and it differs from ours. In their model, the bundle of presumed helices that form the putative Ca^{2+}-binding domain are oriented with the negative charges on the outside of the bundle. Rotation of the helices to internalize the ions is suggested as a subsequent step after both Ca^{2+} ions are bound. The "jaw-closing" motion proposed here is perhaps simpler. It follows more directly from general proposals that have been made in the past for protein-mediated ion translocation across membranes (2, 4, 28–30).

On the other hand, our own model could be incompatible with one feature of the postulated domain map of MacLennan and coworkers. The latter contains helices in the Ca^{2+}-binding stalk domain that are uninterrupted extensions of hydrophobic helices of the transmembrane domain. It is not clear whether that would permit the existence of a hinge at the bottom of the Ca^{2+}-binding cavity, as required by our model.

A particular attraction of our model is that it has a rigidly fixed stoichiometry in terms of number of ions transported per reaction cycle: the closing of the jaws in Fig. 1 and the subsequent ion-translocation step are clearly not possible unless precisely two Ca^{2+} ions are bound. Whether or not the sarcoplasmic reticulum pump actually has a fixed transport stoichiometry (in terms of Ca^{2+}/ATP) is still controversial (31–33), and there is no intention to address that question here. What is important is that there have not previously been any convincing working models for achieving fixed stoichiometry. We have provided such a model, and it gains credibility by the fact that it was not our intended goal to do so.

An equally important feature is that our model suggests a plausible structural basis for direct coupling between ATP processing and ion transport. The transfer of the terminal phosphate group of bound ATP to the protein's phosphorylation site depends on the closing of the jaws of the Ca^{2+}-binding cavity, just as ion translocation does. The model also automatically makes occlusion of bound transport ions a part of the mechanism.

The flexibility of the hinge-closing mechanism should also be mentioned. It is readily adapted to fixed stoichiometries other than 2:1, such as the three Na^+ per cycle in the ATP-driven Na^+/K^+ pump or the Ca^{2+}/Na^+ exchanger. Occlusion of transported ions is an established phenomenon in the former.

A final advantage of our model is that it does not require modification of the basic idea of alternating access, as first proposed by de Meis and Vianna (1), as the intrinsic means of coupling the chemical potential change of the bound Ca^{2+} ions to ATP hydrolysis or synthesis. Our proposed change in the model, ordered Ca^{2+} binding, affects events that occur entirely in one of the alternating access states, when both binding sites face the cytoplasmic side of the membrane. The translocation step is unaffected.

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