TWENTY QUESTIONS CONCERNING THE REACTION CYCLE OF THE SARCOPLASMIC RETICULUM CALCIUM PUMP

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I. INTRODUCTION

Understanding the mechanism of active transport is an intriguing problem[1] because the transported ion does not undergo any chemical change. It enters the transport protein on one side of the membrane at a low chemical potential and leaves on the other side, chemically unchanged, but at a much higher potential — more than 6 kcal/mol higher in the case of Ca^{2+} transported by the sarcoplasmic reticulum Ca pump. In some examples (e.g., Na^+/Ca^{2+} exchange), there is no possibility for direct contact between the ion and the donor that provides the free energy for uphill transport, and even where direct contact is conceivable, as is true for ATP-driven pumps, it is difficult to see how it can be helpful. How does the transport protein accomplish its task?

Solution of this problem can be divided into three sequential parts: (1) specification of the overall catalyzed reactions; (2) specification of the sequential steps of the catalytic reaction cycle that lead to the overall reaction; and (3) analysis of the reaction cycle, with the aid of related structural parameters, to arrive at a mechanism for ion translocation and for free-energy transduction.

The sarcoplasmic reticulum Ca pump is undoubtedly the best understood active transport system at the present time. There are many excellent reviews,[2-7] and a superficial reading of them suggests that the overall problem may in this case be already solved, at least in outline form. The "solution" may be summarized as follows:

1. The protein contains only a single kind of polypeptide chain, with molecular weight about 115,000. It is designed specifically to catalyze the reversible reaction:

$$ATP + 2\ Ca^{2+}\ (cyto) \rightleftharpoons ADP + P_i + 2\ Ca^{2+}\ (SR) \qquad (1)$$

   where "cyto" and "SR" refer, respectively, to the muscle cell cytosol and to the sarcoplasmic reticulum lumen.
2. The reaction cycle, accepted with minor variations by most investigators, is shown in Figure 1. It involves two distinct conformational states of the protein. State E has high-affinity binding sites for two Ca^{2+} ions, accessible only from the cytoplasm, and it also has a high-affinity binding site for ATP. A "high-energy" phosphoenzyme intermediate, $Ca_2E \sim P$, is formed (with dissociation of ADP) when all three sites are filled. The second state is here designated as E′: it also has two Ca binding sites, but they are accessible only from the sarcoplasmic reticulum lumen and their binding affinity is weak. E′ can react reversibly with P_i to form a "low-energy" phosphoenzyme, $E' - P$, which can form the complex $Ca_2E' - P$ if the luminal Ca^{2+} concentration is sufficiently high.

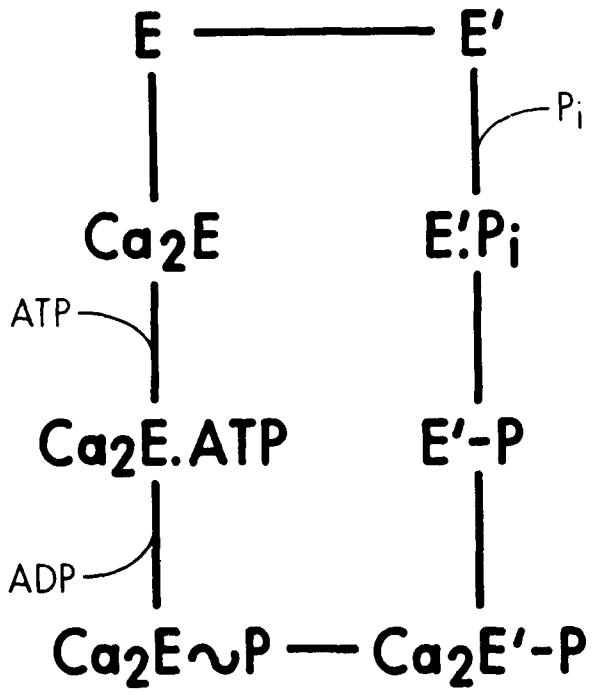


FIGURE 1. Conventional reaction cycle for the sarcoplasmic reticulum Ca pump.[4] The cycle is reversible. Ca^{2+} binds from the cytoplasmic side of the membrane in state E and from the sarcoplasmic reticulum lumen side in state E'. Required participation of Mg^{2+} in some of the steps is not shown.

3. The transport mechanism deduced from this scheme, primarily by deMeis and coworkers,[4,8] is satisfyingly simple. The critical step is the step $Ca_2E \sim P \rightleftharpoons Ca_2E' - P$. The ion binding sites, with bound Ca^{2+} attached, are translocated across the membrane in this step, and there are synchronous changes in the chemical potentials of the bound ligands: the chemical potential of bound Ca^{2+} goes up (high to low binding affinity); the chemical potential of the covalently bound phosphoryl group goes down ($E \sim P \rightarrow E' - P$). Free energy transduction thus appears to be accomplished in a single step by direct exchange between the two bound ligands. A speculative structural model showing how the chemical potential of bound Ca^{2+} might change in synchrony with translocation has been proposed.[9]

A review of the recent literature indicates, however, that most of these aspects of the transport mechanism cannot be considered as firmly established, and the purpose of this paper is to focus on the questions that have been raised. An exhaustive literature review was not attempted, but it is hoped that a sufficient number of investigations have been cited to clearly define the many problems that remain unresolved.

II. OVERALL REACTION

Biochemists are accustomed to dealing with enzymes that catalyze highly specific reactions. Hexokinase, for example,[10] catalyzes the reversible formation of glucose-6-phosphate from ATP and glucose. Isomeric products are not formed, nor is there transfer of a second phosphoryl group to the initial product. The reaction glucose-6-phosphate $\rightleftharpoons$ glucose + P_i

is not catalyzed. Catalysis of uncoupled hydrolysis of ATP has been reported, but the maximal rate observed was less than $1/10^4$ of that of the principal reaction. Do active transport proteins resemble enzymes in this sense of being precisely tailored to perform a highly specific task? If so, what is the catalyzed reaction?

A. Does the Pump Protein Catalyze a Specific Reaction?

Most investigations start out with the assumption that the Ca pump protein catalyzes a precisely specified reaction, but some do not. The theoretical treatment of Jencks,[11] for example, assumes that free energy coupling involves fixed stoichiometry and he has formulated a set of "rules" that are required to ensure it. The rules are essentially restrictions on the composition of complexes undergoing important transitions, such as the $Ca_2E \sim P \rightleftharpoons Ca_2E' - P$ transition of Figure 1. On the other hand, Hill[12] has theoretically discussed ATP-driven ion pumps in terms of a model that explicitly assumes that the interconversion between the E and E′ states of Figure 1 can occur in any binding state of the protein. This leads to the possibility of several different reaction cycles, each catalyzing a different overall reaction. If his model is applied to the Ca pump reaction, it would permit four different overall reactions to occur: two coupled reactions with stoichiometries of two Ca^{2+} and one Ca^{2+}, respectively, per ATP, and also uncoupled ATP hydrolysis and downhill passive transport of Ca^{2+}. There would be no unique overall stoichiometry: it would be dictated by relative rates of the different possible cycles, which would in turn be a function of external conditions.

Most of the experimental evidence (within the limits of accuracy of measurements of transport stoichiometry) supports a fixed stoichiometry of two Ca^{2+} per ATP, not only for active transport, but also in the reverse direction for ATP synthesis.[7] Moreover, the physiological steady state in a resting cell is close to that predicted for the equilibrium state of Equation 1 with fixed stoichiometry.[13] Observed changes in measured coupling ratio at high pH or temperature probably reflect changes in passive membrane permeability rather than changes in pump stoichiometry.[7] However, an old report of reduced transport stoichiometry at low temperature[14] may not be explainable in the same way, and a more recent report[15] suggests that phosphorylation of one protein molecule by GTP can induce ion transport in neighboring molecules, leading to a transport stoichiometry of >2 Ca^{2+} per GTP. A reassessment of these data[7] suggests a somewhat different interpretation, but the new interpretation still involves variable stoichiometry in at least a part of the reaction cycle.

A related question can be asked: if the transport reaction has a fixed stoichiometry, is it because the reaction occurs by an ordered sequence of discrete purposeful steps that could lead to no other result? Analogy with other enzyme-catalyzed reactions (e.g, hexokinase) would suggest an affirmative answer to this question, and an affirmative answer is implicit in the reaction scheme of Figure 1. Berman,[16] however, has expressed a contrary opinion. He points out that disruption of the reaction cycle by mild denaturation of the pump protein often leads to loss of transport function, but retention of ATPase activity. He proposes that a pathway for uncoupled ATP hydrolysis might well exist even in the native protein, even though it may not contribute significantly to the overall reaction stoichiometry under physiological conditions. This proposal is not trivial in relation to the reaction mechanism: if Berman is right, then Figure 1 is conceptually misleading in its suggestion of a strictly ordered mechanism.

The conclusion from these data has to be that a fixed reaction stoichiometry is not yet unambiguously established.

B. Is Counter-Transport of Other Ions an Integral Part of the Pump Reaction?

A more serious problem is that assumption of a constant Ca^{2+}/ATP stoichiometry does not by itself imply that Equation 1 is an adequate representation of the overall reaction. The

equation as written calls for translocation of four positive charges per reaction cycle, and in the absence of compensatory movement of other ions this would lead to rapid buildup of a membrane potential that would bring the reaction to a halt (following muscle contraction) before the pump has been able to remove an adequate amount of Ca^{2+} from the cytoplasm for muscle relaxation to occur. The sarcoplasmic reticulum membrane is highly permeable to Na^+, K^+, and to many anions, so that there is no apparent physiological need for charge compensation by any other pathway. On the other hand, there may be a need for compensatory ion movement within the pump protein itself to overcome activation barriers in translocation steps (see Section VIII.C.), or compensatory movement may exist for evolutionary reasons. For example, the overall reaction of ATP-driven Na, K pumps involves the transport of three Na^+ out of the cytoplasm and the obligatory counter-transport of two K^+ into the cytoplasm. The sarcoplasmic reticulum Ca pump protein may have evolved from the Na,K pump proteins (there are striking similarities in the reaction cycles), and may have retained obligatory K^+ counter-transport as part of its reaction sequence. Possible similarity between the sarcoplasmic reticulum Ca pump and the Ca pump of plasma membranes would constitute another possible reason for counter-transport as an integral part of the reaction sequence because plasma membranes do not have the passive pathways for charge compensation that exist in the sarcoplasmic reticulum membrane. In any event, the focus in this review is on the reaction mechanism and the existence of mandatory counter-transport would have an important effect on theoretical models for how ion translocation is accomplished.

The inherent leakiness of the sarcoplasmic reticulum membrane makes it difficult to measure counter-transport in sarcoplasmic reticulum vesicles, and experimental problems involved in the reconstitution of purified pump protein into tight artificial vesicles have so far prevented the acquisition of unambiguous data by that route. Investigators who have addressed the question come to widely differing conclusions. Kasai[17] concludes that all movement of ions across the sarcoplasmic reticulum membrane (other than Ca^{2+}) is a passive response to the generation of a membrane potential and that there is no mandatory counter-transport by the pump itself. At the other end of the scale, Chiu and Haynes[18] conclude that the overall pump cycle is probably electroneutral by virtue of counter-transport of 4 K^+ ions. This latter conclusion would seem to be untenable: experiments in reconstituted vesicles are sufficiently advanced to demonstrate whether or not the pump reaction is electrogenic and the results obtained all agree that it is.[19-22] This of course leaves open the possibility of partial charge compensation, with K^+ the most viable candidate for counter-transport if it occurs. Chiesi and Inesi[23] have shown that Mg^{2+} counter-transport is unlikely: Mg^{2+} is an essential co-factor for formation of the phosphoenzyme complexes, but the bound phosphate is not translocated, so that this role of the ion does not require or even suggest the likelihood of Mg^{2+} transport across the membrane. (Co-transport of anions has sometimes been speculatively proposed, but the available evidence is that anion transport occurs via separate passive channels[24]).

Counter-transport of H^+ represents a special problem. Any protein binding site for Ca^{2+} is bound to contain carboxyl groups (more than one if the affinity is high). These groups, in their free state, are likely to be partially protonated around pH 6, and, if there are two or more of them close together, protonation would persist to pH 7 or above. This phenomenon could well lead to the translocation of protons in the step $E \rightleftharpoons E'$ of Figure 1, without counter-balancing translocation in the step $Ca_2E \sim P \rightleftharpoons Ca_2E' \sim P$, where Ca^{2+} would have replaced the H^+ ion bound to the carboxyl groups, i.e., it could formally lead to experimentally observable net H^+ transport for what might be called trivial reasons. Counter-transport of H^+ as part of the pump reaction has in fact been observed,[23,25,26] but the stoichiometry is variable, becoming minimal at neutral pH in a manner consistent with a "trivial" mechanism of this kind. The nontrivial possibility that there might be mandatory H^+ counter-transport related to physiological function or to the basic pump mechanism (e.g.,

the possibility that the transition state for one of the conformational transitions cannot exist without incorporation of an integral number of H^+ ions) does not appear to be supported by existing data. It should perhaps be mentioned that the evidence for occurrence of any kind of H^+ counter-transport through the pump protein has been questioned.[27]

III. SITE STOICHIOMETRY

It is somewhat surprising to find that the intrinsic stoichiometric properties of the Ca pump protein remain in question. It is now generally accepted that all the functional properties of the pump reside within a single kind of polypeptide chain, but even this cannot be said to be truly established. There has been a loss of interest in the "proteolipid" polypeptide of low molecular weight that is often found associated with preparations of purified pump protein, but its complete irrelevance to structural or functional properties has not been proved.[28] Assuming that the polypeptide of molecular weight about 115,000 is the only relevant constituent of the protein, the major unresolved questions are the number of functionally important binding sites per chain and whether the physiologically functional protein is a monomer or an oligomer. These questions are separate questions in terms of direct experimental approach, and will be treated as such here, but they are to some extent interrelated, e.g., if there is only one Ca^{2+} binding site per chain, then at least two chains are required to account for a transport stoichiometry of two Ca^{2+} per reaction cycle. (The alternative possibiity that a single chain might go through two successive cycles for each ATP hydrolyzed is not compatible with kinetic data.)

Knowledge of the content of pump protein in the preparation being used is essential for the establishment of site stoichiometry. Pickart and Jencks[29,30] found that the vesicular preparations they obtained by a slight modification of the popular MacLennan procedure[31] were contaminated by glycogen phosphorylase b, and thereore contained significantly less pump protein than is generally assumed to be present in such preparations. This could account for some of the existing confusion about site stoichiometry, but it cannot account for all of it: Watanabe et al.,[32] e.g., obtained similar data for the number of Ca^{2+} binding sites per polypeptide chain, independently of whether they used the MacLennan procedure or the preparative procedure of Meissner,[33] but their result differs from that obtained by Meissner himself.

A. How Many Polypeptide Chains per Functional Unit?

It has been demonstrated[34-36] that the Ca pump protein can be solubilized in the nonionic detergent dodecyl octaethyleneglycol monoether ($C_{12}E_8$) and that it exists in the solution as a monomer. Use of the method of active enzyme centrifugation showed that the monomer is the catalytic unit for ATP hydrolysis[35] and subsequent work[37] has demonstrated that all the partial reactions of Figure 1 can be carried out in both directions in the monomeric state. Transport per se cannot of course be measured in a homogeneous solution, but the fluorescence change that is generally assumed to reflect the $E \rightleftharpoons E'$ conformational change can be observed, and the Ca^{2+} binding sites in the two states show the same dramatic difference in binding affinity as in vesicular preparations. The protein is, however, much less stable in the soluble state, and there are significant quantitative differences in the reaction between E' and P_i or ATP. There are many possible explanations for these differences (e.g., a specific lipid requirement, see Section VII.A), and a difference in the state of polypeptide association is one possibility. It should be noted that measurements made so far have not conclusively proved whether the strict 2:1 (Ca^{2+}/ATP) transport stoichiometry or the cooperativity of Ca^{2+} activation of the pump are maintained in solution, so that a possible requirement for an oligomeric structure to elicit the optimal physiological response to changing Ca^{2+} concentration has to be considered.

Direct structural studies have mostly indicated that the pump protein exists as an oligomer in the sarcoplasmic reticulum membrane and in reconstituted vesicles,[38-40] but an X-ray scattering study by Brady et al.[41,42] indicates that the dominant species in the membrane is monomeric. Energy transfer or excimer formation for pump protein with an attached fluorescent label has suggested extremely close association between monomers in the membrane;[43,44] excimer formation disappears on solubilization of the protein by detergent in a presumably monomeric state.[44] Data of this kind do not necessarily provide conclusive evidence regarding the size of the functional unit, since functionally independent molecules could be in an associated state in the membrane for purely physical reasons, as is demonstrated by the fact that bacteriorhodopsin exists as a crystalline sheet in native purple membranes, though it is functionally monomeric.[45]

Measurement of the target size for destruction of biological actvity is more directly related to function, but results from this type of measurement are again inconclusive. The target size for radiation inactivation corresponds to a dimer,[46] which means that a "hit" on any one polypeptide chain leads to inactivation of two chains. On the other hand, individual polypeptide chains respond independently to inactivation by covalent binding of fluorescein, and response to this type of inactivation is the same for $C_{12}E_8$ solubilized and membrane-bound protein.[47] An earlier report that 1 mol of bound fluorescein inactivates two chains[48] has proved to be incorrect.[49]

Thermodynamic or kinetic cooperativity can, in principle, provide unambiguous evidence for participation of more than one polypeptide chain in the binding of a ligand. Watanabe et al.[32] observed that the Hill coefficient for equilibrium Ca^{2+} binding in the absence of ATP is pH dependent: the coefficient has a value of about 2.5 at physiological pH and a value as high as 3.3 was found at pH 8.5. This requires a binding stoichiometry of at least four Ca^{2+} per functional site (>2 even if the result at pH 8.5 is discarded). The same paper concluded that there is only one Ca^{2+} binding site per chain, which means that the minimal functional unit would have to consist of four polypeptide chains. If there are actually two Ca^{2+} binding sites per chain (see below), the minimal functional unit would still have to be at least a dimer. Kinetic data and cross-linking studies of Ikemoto and co-workers[50-52] also indicate an oligomeric functional unit: they specifically suggest that the pump cycle involves two kinetically distinguishable subunits that are out of phase as they carry out the sequential steps of the reaction cycle. This suggestion (and, more generally, any functional requirement for an oligomeric structure) of course implies that the reaction cycle of Figure 1 is fundamentally incorrect.

B. How Many Substrate Processing Sites per Chain?

The rate of ATP hydrolysis has a biphasic dependence on ATP concentration, which was initially interpreted as indicating the existence of two distinct, independent ATP binding sites per functional unit, one of them being the catalytic site and the second one being a separate low-affinity activator site. Neet and Green[53] showed that these results could equally well be explained in terms of a single site having different affinities in two states of the reaction cycle. This explanation would be consistent with the conventional reaction scheme given in Figure 1. The scheme is based on a two-conformation model and the phosphoryl group is known to remain attached to the same aspartyl residue in the transition from $E \sim P$ to $E' - P$, although the properties of the group change dramatically. It is therefore reasonable to expect that the substrate binding site should also have different affinities in E and E′ (both absolute and relative) for ATP, P_i, and analogs of these ligands. In particular, it is reasonable that the affinity for ATP should be lower in E′ because phosphorylation from ATP occurs only in state E. All that would then be needed to explain rate acceleration at ATP concentrations beyond what is required for saturation of the high-affinity site on E is to suppose that the conformational transition $E \rightleftharpoons E'$ is inherently slow and rate determining

for the overall reaction, and that it becomes faster when the low-affinity site on E′ is occupied. A similar situation exists for ATP-driven Na,K pumps, and a similar single site explanation has been proposed.[54] In both cases, however, the single-site explanation remains controversial, and it has not been demonstrated that it can account quantitatively for experimental data. The possible existence of a separate activating site has thus not been excluded.

A second point of controversy arises from discrepant results for the maximal amount of phosphoenzyme ("EP" = $Ca_2E \sim P + Ca_2E' - P + E' - P$ in terms of Figure 1) that can be formed when the high-affinity sites for Ca^{2+} and ATP on E are saturated. Results that indicate formation of close to one EP per chain (within the limits of error of estimating pump protein concentration) have been reported,[33,39] but the more common result is 0.5 EP per chain (e.g., Inesi et al.[55]). If the latter result is correct, it could imply a mechanism involving half-of-the-sites' reactivity, e.g., out-of-phase cycling of two subunits as suggested by Ikemoto and co-workers.[51] On the other hand, the result need not be inconsistent with the reaction scheme of Figure 1. The measured EP value is a steady-state value and the kinetic parameters of the reaction cycle may limit the maximal EP level that can be obtained during cycling of the protein (see related discussion in Section VI.B).

An obvious prerequisite for resolving these important mechanistic questions is an accurate count of the number of substrate binding sites per polypeptide chain. ATP itself, other nucleotides, and a fluorescent derivative (TNP-ATP) have been used for the measurement. If the pump protein contains only a single kind of polypeptide chain, as generally assumed, there cannot be less than one site per chain (nor less than one aspartyl residue for EP formation). Most measurements in fact indicate one site per chain[56,57] within the range of free ligand concentration that was investigated. The data do not preclude the existence of a second site with low affinity, i.e., they cannot, with any confidence, be used to exclude the possible existence of a separate activation site. A more serious problem with the data, however, is the relation between the number of ATP binding sites and the number of high-affinity Ca^{2+} binding sites: Meissner[33] reported two Ca^{2+} sites per ATP site, but Dupont et al.[56] and Inesi and co-workers[57,59] found equal numbers of sites for the two ligands. If there are, in fact, two Ca^{2+} binding sites per polypeptide chain (see below) the measurements from these two laboratories would require two nucleotide binding sites of relatively *high* affinity per chain. No conceivable functional reason for two high-affinity sites has been suggested. When half-of-the-sites reactivity schemes for free energy coupling are proposed (e.g., Ikemoto et al.[51]), it has always been done in terms of one ATP site per chain and two chains per functional unit, and not in terms of two ATP sites on the same chain.

C. How Many High-Affinity Ca Sites per chain?

The sites that pick up Ca^{2+} from the cytoplasmic side of the membrane reside (by definition) in the E state of the protein. They must have a very high affinity for Ca^{2+}, as a consequence of which all the pump protein becomes converted to the E state when the free Ca^{2+} concentration exceeds the physiological resting state concentration ($<10^{-7}$ *M*) by two or three orders of magnitude: in the absence of ATP, where no cycling can occur, virtually all of the protein must be present as the complex between E and Ca^{2+}. This suggests that the number of Ca^{2+} binding sites per polypeptide chain should be easy to measure and that it should be one of the uncontroversial properties of the pump protein. The available data unfortunately do not bear this out.

Meissner[33] reported two high-affinity Ca^{2+} binding sites per polypeptide chain. Ikemoto[58] reported two sites at 22°C, but found that one of the sites had greatly reduced affinity when the temperature was reduced to 0°C. The latter result was consistent with an earlier claim by Sumida and Tonomura[14] that the Ca^{2+}/ATP transport stoichiometry changes from 2:1 to 1:1 as the temperature is reduced from 22 to 0°C, and an effect of temperature within this range is consistent with a break near 20°C in the Arrhenius plot for ATP hydrolysis. This

break is generally assumed to reflect a protein conformational change (presumably not related to $E \rightleftharpoons E'$) because it occurs for detergent-solubilized pump protein as well as in the membrane-bound form[34] and therefore cannot be due to a phase transition in the lipid bilayer, an explanation often suggested for this and other anomalous temperature dependencies of reactions catalyzed by membrane-bound proteins.

More recent data throw doubt on these results. Inesi and co-workers[59] find only one site per chain by direct titration of Ca pump protein in vesicular form. Though the binding affinity is pH dependent, the binding capacity is not. Moreover, the binding isotherm near pH 7 was found to be independent of temperature, which is in conflict with the data of Ikemoto cited above. Silva and Verjovski-Almeida[60] have used a similar direct binding technique to compare monomeric protein in $C_{12}E_8$ solution with vesicular pump protein. They also found that there is only one site per chain in the vesicular state, but the binding data for soluble protein correspond to two sites. They speculatively explain this by suggesting that one of the two sites is not accessible in the vesicular state. An alternative possibility is that the pump protein in vesicular preparations is less pure than is generally supposed (see above). Murphy,[61] also using the same general procedure, has measured maximal binding of only slightly more than one Ca^{2+} per chain to $C_{12}E_8$-solubilized monomeric protein, but the binding isotherm was found to be highly cooperative, with a Hill coefficient close to two. This result is incompatible with the presence of fewer than two sites per molecular particle and, since the particle is in this case a monomer, requires at least two sites per chain. The low value for maximal binding suggests that in this case the soluble protein may have been contaminated by impurities or inactive protein. (Interpretation of these results is further confused by the observation of Silva and Verjovski-Almeida[60] that the binding of Ca^{2+} to the monomeric form of the protein is *not* cooperative, even though *their* maximal binding value corresponds to two sites per chain.)

IV. NUMBER OF CONFORMATIONAL STATES

In the scheme of Figure 1 the pump protein alternates between two conformational states, with Ca^{2+} binding sites facing opposite sides of the membrane. It is difficult to conceive of any mechanism for active transport of Ca^{2+} without at least these two distinct conformational states.[62] There is unambiguous evidence for two conformational states for Na,K pumps, because the initial products of tryptic hydrolysis are different in the presence of excess Na^+ and excess K^+, which is consistent with the high probability that high-affinity uptake sites for these two ions should be on opposite sides of the membrane.[63] A change in intrinsic protein fluorescence occurs in parallel with the change in hydrolysis pattern. For the Ca pump protein no differences in tryptic hydrolysis patterns have been reported, but it is firmly established that the presumptive conversion of all of the protein to the E state upon Ca^{2+} binding is accompanied by a change in intrinsic fluorescence, similar to that found for Na,K pumps.[64,65] These considerations suggest that the existence of two conformational states is reasonably certain. What is uncertain, however, is whether they are the only significant conformational states that occur in the course of a reaction cycle. This section of the paper will address this question and will also discuss the meager existing evidence on structural and thermodynamic differences between the two conformations that we are accepting as reasonably well established.

Enumeration of additional conformational states requires a definition of what is meant by "significant conformational change", and there exist no generally accepted rules for that. It has to be recognized that a protein molecule in a given state does not have a rigidly fixed structure, but fluctuates between numerous microstates of about equal free energy: these fluctuations are normally regarded as attributes of any definable single state and changes in the distribution between different microstates (e.g., as a function of pH) are not treated as

a conformational change. Similarly, it is hardly conceivable that binding of a ligand to a preexisting binding site can occur without some rearrangement within the binding area: common sense again dictates that this kind of change should not be regarded as a significant conformational change, but that it should be treated as analogous to movement of solvent molecules within or near the binding site, i.e., simply as part of the reaction ligand + empty site $\rightleftharpoons$ occupied site. At the other extreme, changes in long-range order or changes in accessibility of side-chain groups (e.g., SH or peptide groups) to chemical reagents are unambiguous evidence for conformational change. Changes in the fluorescence spectrum and other spectral changes are commonly used as indicative of conformational change, but this is not a really safe assumption. It is possible that the binding of a ligand to a fixed site can alter the environment of a nearby tryptophan residue, for example, sufficiently to affect the fluorescence spectrum even in the absence of a change in protein structural parameters outside the binding site. The red cell glucose transporter has been cited as an example.[66] In the absence of extensive structural data, such as exist for hemoglobin, the decision as to whether or not there is a significant conformational change accompanying some step in a reaction cycle can sometimes not be made with confidence.

A. Is there an Occluded State with Binding Sites Inaccessible from Either Side of the Membrane?

There is good evidence that bound Ca^{2+} in the state $Ca_2E \sim P$ of Figure 1 cannot be rapidly released to the solution on either side of the membrane:[67-69] the rate of dissociation from this form of the protein is much lower than the rate of dissociation from Ca_2E or the rate of release to the sarcoplasmic reticulum lumen after translocation of the binding sites has occurred in the step $Ca_2E \sim P \rightleftharpoons Ca_2E' - P$ (Figure 1). The simplest interpretation is that the part of the protein involved in ion translocation exists in $Ca_2E \sim P$ in a state intermediate between E and E′, in which the bound ions (both of them, simultaneously) are "occluded", i.e. not readily accessible from the aqueous media on either side of the membrane. Such an alteration would presumably fall within the definition of a conformational change, but direct support for this, e.g., from fluorescence measurements,[65,70] is lacking.

It is of interest in this connection that there is good evidence for the existence of occluded bound K^+ in the reaction cycle of ATP-driven Na,K pumps. The conventional reaction cycle for this pump is similar to that of Figure 1, with Na^+ replacing Ca^{2+} and with K^+ transported across the membrane as part of the transition $E' \rightleftharpoons E$: K^+ appears to be occluded in the E′ state, and dissociates from its binding site only after conversion to state E.[71] As noted earlier, it is possible that the Ca pump mechanism may involve similar K^+ counter-transport. (It has also been suggested that bound Na^+ may be occluded in the $Na_3E \sim P$ state that corresponds to $Ca_2E \sim P$ in Figure 1.)

An important point to be made here is that the existence of an intermediate state in the translocation of the binding sites from one side of the membrane to the other (which in Figure 1 occurs by definition in the $E \rightleftharpoons E'$ and $Ca_2E \sim P \rightleftharpoons Ca_2E' - P$ transitions) need have little effect on the conceptual mechanism for ion translocation and free-energy transduction, especially if the intermediate can be shown to occur in the unphosphorylated form of the protein as well as the phosphorylated form. If the chemical potential of occluded Ca^{2+} (both bound ions) is the same in $Ca_2E \sim P$ and $Ca_2E.ATP$ (Figure 1), then the intermediate state would reflect nothing more than the physical inability to move the binding site from one side of the membrane to the other in a single step.

B. Is There a State in Which the Two Ca Binding Sites are Accessible from Opposite Sides of the Membrane?

Detailed studies[72,73] of the transition from unliganded protein to the state Ca_2E, induced by binding of Ca^{2+}, suggest the possibility of a fundamental flaw in the reaction scheme

of Figure 1, and thereby a need for serious revision of the transport mechanism suggested by it. In the scheme of Figure 1, the unliganded protein would be present largely in the form E′, which, by definition, has both Ca^{2+} binding sites in their low-affinity form, facing the sarcoplasmic reticulum lumen, whereas the product Ca_2E has both sites in their high-affinity form, facing the cytoplasm (the "outside" in experiments with sarcoplasmic reticulum vesicles). The fluorescence change that accompanies the formation of Ca_2E is normally assumed to reflect the structural change of the protein as it goes from state E′ to state E; the reaction scheme of Figure 1 would require this change to occur before any Ca^{2+} can be bound to the protein from the cytoplasmic side. The detailed studies cited here, however, suggest that one of the two Ca^{2+} binding sites is already accessible from the cytoplasmic side in the unliganded state and that the conformational change to state E occurs only after this site is occupied. Since there is ample evidence that both Ca^{2+} binding sites face the sarcoplasmic reticulum lumen in the state $Ca_2E' - P$ (and it is difficult to imagine any other possibility), these results require a new conformational state with the two binding sites facing opposite sides of the membrane. The existence of this kind of state would require a much more serious revision of the scheme of Figure 1 than would the occluded states discussed in the previous section.

Dupont[65,72] has pointed out that if the functional unit of the pump protein is a coupled dimer of two subunits, each of which contains two transport sites, one can account for these results in terms of coupling between subunits and need not invoke the existence of a state in which the two Ca^{2+} binding sites of an individual subunit face opposite sides of the membrane. However, as previously pointed out, an oligomeric functional unit of this kind would itself require revision of the conventional transport mechanism given in the Introduction. In fact, unless the experimental data are due to some artifact (e.g., the work of Dupont[72] was done at very low temperature), it is difficult to imagine any way to reconcile these results with the scheme of Figure 1. One rather far-fetched possibility is to suppose that the transport protein is unstable in the state E′, and that it undergoes a slow transition to a reversibly denatured state if it resides in the state E′ for a significant length of time, which it does in the present experiments because this is the initial state for the measurements. In that case, the slow and fast steps of the biphasic response to addition of Ca^{2+} (on which the conclusions of the studies cited here are based) might reflect a slow "renaturation" plus a single functional step rather than two successive functional steps of the normal reaction cycle. A similar explanation has been previously suggested[74] to account for anomalous calorimetric data that had been obtained in a study of the conversion of E′ to $E' - P$.[75]

C. What is the Magnitude of the Structural Change in the $E \rightleftharpoons E'$ Transition?

Even a crude knowledge of the structural change involved in the $E \rightleftharpoons E'$ transition (and other conformational transitions, if they exist) would significantly aid our understanding of the transport mechanism, but there are no useful experimental data on this subject. Differences in fluorescence spectrum or accessibility of a peptide group to proteolysis do not necessarily imply that there has been a major rearrangement of the overall protein structure; in fact, extensive unfolding and refolding would probably be slow processes that could not be part of a reaction cycle that has to proceed at a sufficiently high rate to meet physiological requirements. A speculative paper by the author of this review[9] has suggested that alternating access of the Ca^{2+} binding sites to opposite sides of the membrane, with a synchronous change in the binding constant, does not necessarily require a major rearrangement of the protein structure: a specific model was suggested (based on the known structure of the transmembrane domain of bacteriorhodopsin) to show that the requisite changes could be produced by a small translational and rotational realignment of more or less parallel transmembrane helices, without need for significant change in polypeptide backbone structure. This suggestion should be directly testable by measurement of the change in circular dichroism spectrum associated with the $E \rightleftharpoons E'$ transition.

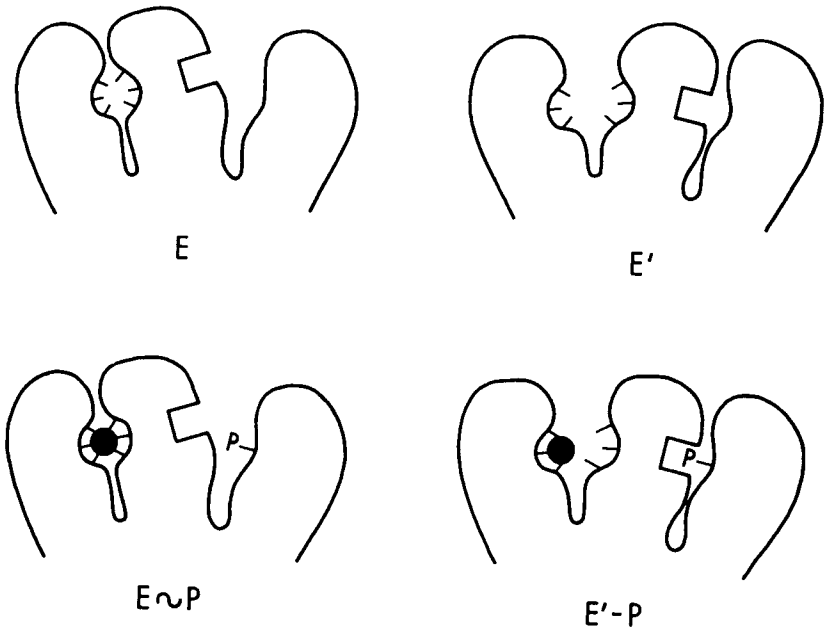


FIGURE 2. Schematic double-hinge model to show how a protein can transfer free energy directly from a "high energy" phosphate group to a bound Ca^{2+} ion (black circles) by means of a synchronous increase in the chemical potential of one ligand at the expense of a decrease in the other. E and E′ are assumed to have roughly equal free energies. The short lines in the Ca^{2+} binding site represent chelating groups. The kind of groups (in the square notch) that might affect the chemical potential of the bound phosphate are not yet known (see Section VI.A).

If the apparent occlusion of the Ca^{2+} binding sites in the state $Ca_2E \sim P$ is taken as indicative of an overall conformational difference between this state and $Ca_2E.ATP$, there is again no compelling reason to think that the difference must involve a major refolding. "Hinge bending" is a structural change that has been observed for several water-soluble enzymes, and it involves a movement of two domains of the enzyme protein towards each other without rearrangement of the domains themselves. In one of the best-known examples, yeast hexokinase,[76] the bending movement closes a cleft in the structure by which glucose has access to its binding site. This is a conceivable model for what might be occurring in the Ca pump protein.

D. What is the Free-Energy Difference Between the E and E′ States?

The free-energy difference between conformational states of the protein is a crucial parameter for elucidation of the free-energy transduction mechanism. Two extreme possibilities for free-energy transduction have been suggested: Lumry[77] suggested that the free-energy derived from ATP hydrolysis can be used to drive the pump protein into a thermodynamically unfavorable state and that the free-energy released by return of the protein to its original state can be used in a separate step to increase the chemical potential of the transported ions; the author of this review[78] suggested the possibility of a direct transfer mechanism in which the protein is energetically inert (different protein conformations have about the same free energy) and the chemical potential of the transported ions goes up at the expense of the chemical potential of bound phosphate in a single step. Figure 2 shows schematically how this might be accomplished. The reaction scheme of Figure 1 is consistent with a direct transfer mechanism if the bound Ca^{2+} in state $Ca_2E \sim P$ has the same low chemical potential

as in state Ca_2E (high binding affinity corresponds to a low chemical potential in the bound state). The chemical potential of bound phosphate is about 10 kcal/mol lower in $Ca_2E' - P$ than in $Ca_2E \sim P$ and the chemical potential of each bound Ca^{2+} in $Ca_2E' - P$ must be about 6 kcal/mol higher than in Ca_2E in order to account for rapid release from that state into the sarcoplasmic reticulum lumen.[4,79] $\Delta G^{o\prime}$ for the overall transition $Ca_2E \sim P \rightleftharpoons Ca_2E' - P$ is about zero,[8,80] which implies that little free energy can be contributed by the protein structural rearrangement per se.

If this is true, and if E and E′ are the only functionally important states of the protein, then $\Delta G^{o\prime}$ for the reaction $E \rightleftharpoons E'$ should also be close to zero. This means that E and E′ should be present in about equal concentrations in the absence of ligands that bind differentially to the two states, and this in turn means that the equilibrium constant for their interconversion should be readily measurable by use of spectral parameters that can be assigned absolute values characteristic of the two states. The intrinsic protein fluorescence (discussed earlier) would in principle be suitable for this.

Pick and Karlish[81] have made the appropriate measurements using fluorescein-labeled pump protein. They used vanadate to convert the protein completely into the E′ state and Ca^{2+} to convert it into the E state: fluorescence levels in the absence of either of these reagents led to the conclusion that the E′/E equilibrium is dependent on temperature and pH: at pH 7 and 25°C K_{eq} was found to be close to unity, yielding $\Delta G^{o\prime} = 0$ for the transition between the two conformations. This result cannot be regarded as definitive because fluorescein binding inactivates the protein; in fact, the data themselves suggest that the $E \rightleftharpoons E'$ equilibrium has been perturbed because the conversion to Ca_2E was found to be noncooperative with respect to Ca^{2+} concentration, whereas it is always cooperative in similar vesicular preparations of the unmodified protein. If E and E′ are the only conformational states involved, then the normally observed cooperativity in fact requires that E′ be more stable than E by about 3 kcal/mol.[82] Neither of these $\Delta G^{o\prime}$ values would of course represent a meaningful estimate if the $E' \rightarrow Ca_2E$ transition cannot be treated as a two-state transition, a possibility suggested by the data discussed in the preceding section.

Both of the preceding estimates are based on measurements made in the presence of a high concentration of K^+. Loomis et al.[83] have indirectly estimated $\Delta G^{o\prime}$ for the $E \rightarrow E'$ transition in the absence of K^+ (however, at pH 6) on the basis of the degree of cooperativity observed for the inhibitory effect of high Mg^{2+} concentrations on the formation of $E' - P$, again assuming that E and E′ are the only conformations involved. The value obtained (about -4 kcal/mol) is close to the value based on the cooperativity of Ca^{2+} binding in the presence of K^+. This is of interest in relation to the possibility of K^+ counter-transport (Section II.B): if K^+ has different affinities for E and E′, then the observed E/E′ equilibrium should be altered in the presence of K^+.

Although none of the preceding estimates can be regarded as definitive quantitative determinations of the free-energy difference between the E and E′ states of the protein, they are qualitatively self-consistent and support a direct transfer mechanism for free-energy transduction. Even 4 kcal/mol for $\Delta G^{o\prime}$ is much smaller than the total free-energy exchange in the reaction cycle, which amounts to about 14 kcal/mol.[84]

V. Ca BINDING SITES

A. Are the Two High Affinity Sites Significantly Different?

Precise structural information about the Ca^{2+} binding sites and their positions relative to other parts of the protein would clearly be highly desirable. It is unlikely that such information can be obtained for the low-affinity form of the sites in the discharge state (E′ in Figure 1) until the complete three-dimensional structure of the protein is known. In the state E, however, the sites have very high affinity for Ca^{2+}: the exact affinity is unknown for reasons

given in the following section, but an estimate of about 10^8 for the binding constant is not unreasonable. A number of Ca^{2+} binding proteins are known to have binding sites with an affinity of this order of magnitude. They all form octahedral complexes with Ca^{2+} (structurally similar to the Ca^{2+}-EGTA complex), and the sites required to form these complexes appear in all of them to be generated by a similar characteristic sequence of about 30 amino acid residues.[85] If the complete primary structure of the Ca pump protein were known, one could search for segments of appropriate sequence and could perhaps decide with reasonable confidence whether there are one or two sites per polypeptide chain, and (if two) how different they are, whether they might be located in the same binding pocket, etc.

Although 80% of the primary structure of the protein has been determined,[86-88] no sequences suggestive of a typical Ca^{2+} binding sequence have been seen. The undetermined 20% of the primary structure contains more than its fair share of the hydrophobic amino acid side chains, i.e., those parts of the polypeptide most likely to be in direct contact with the core of the lipid bilayer, and this is of course the part of the polypeptide that one would expect to contain the machinery for Ca^{2+} translocation. (The phosphorylation site of the protein has been identified in one of the hydrophilic segments of the amino acid sequence, from which one might speculatively infer that this site is not located near the Ca^{2+} binding sites.) It is also possible that the Ca^{2+} binding sites in this protein do not resemble the binding sites of known Ca^{2+} binding proteins: the sites could be created by close approach of two or more noncontiguous segments of the sequence and that could be the reason why they have not been identified so far.

B. What are the Binding Constants for Ca^{2+} Uptake and Discharge?

It is difficult to conceive how active Ca^{2+} transport can occur at all unless the binding sites have very different affinity for the ion in the uptake and discharge conformations.[79] All available experimental data are qualitatively consistent with this,[4,8] but precise interpretation of the data in terms of quantitative thermodynamic parameters has so far not been possible.

True equilibrium data for Ca^{2+} uptake from the cytoplasmic side of the membrane (the "outside" in vesiculated sarcoplasmic reticulum) can be obtained by omitting ATP and P_i from the medium. Binding isotherms obtained under these conditions are generally found to be cooperative and 50% saturation of the sites is generally achieved (at pH 7) at a free Ca^{2+} concentration well below 1 μM. There are discrepancies in the reported degree of cooperativity and therefore in the minimal number of interacting binding sites that constitute one "binding unit", e.g., an early study from Inesi's laboratory[59] gave a Hill coefficient of 1.82 at pH 6.8, requiring a minimum of two interacting binding sites, whereas a later study[32] reported a Hill coefficient of 2.1 at the same pH and even higher values at higher pH, requiring analysis in terms of at least four interacting binding sites. As previously noted, there are also discrepancies relative to the amount of protein present, leading to different conclusions as to the number of polypeptide chains that correspond to one "binding unit".

It is always possible to describe such data in terms of operational binding constants ($K_{app,i}$), equal in number to the number of interacting sites, but it is well understood that these parameters do not more than describe the data and do not lend themselves to interpretive analysis.[89] The data of Inesi et al.[59] (two sites), for example, can be described in terms of $K_{app,1} = 1.2 \times 10^5 M^{-1}$ and $K_{app,2} = 5 \times 10^7 M^{-1}$, which is just another way of representing the midpoint and degree of cooperativity of the original data. The data of Watanabe et al.[32] (four sites) were described in a slightly different, but equally empirical way: a formal "interaction free energy" was used to describe the change in apparent binding affinity with the extent of occupancy of the binding sites.[90]

For ions of like charge, where an actual attraction between bound ions is inconceivable, the only reasonable way to account for positive cooperativity ($K_{app,i} > K_{app,i-1}$) is to suppose

that one or more binding sites are either not available at all in the unliganded protein and become available only as a result of a conformational change, or that they have a low binding affinity in the unliganded state, which increases as a result of a conformational change.[91,92] The data of Inesi et al.[59] have been analyzed on this basis:[82] the validity of the scheme of Figure 1 was assumed, high-affinity binding sites were assumed to be identical and independent and present only in state E. The data could be accounted for by a true binding constant of about $10^8\ M^{-1}$ to the sites in state E and an equilibrium constant of about 10^3 for the E $\rightarrow$ E′ transition (see Section IV.D). Analysis of the data would become more complicated (and a unique interpretation would probably not be obtained) if the scheme of Figure 1 was discarded and allowance was made for an additional conformational state in which binding sites facing opposite sides of the membrane were simultaneously present (see Section IV.B).

All that can definitely be said about the high-affinity sites, therefore, is that the protein must undergo conformational change as part of the binding process and that the equilibrium constants for binding to the final conformation (i.e., state E) must be higher than the "apparent $K_{1/2}$" that would be obtained from the midpoint of the binding isotherm.

Information about the low-affinity sites is even less precise. One reason is that equilibrium data for low-affinity binding are quite generally difficult to obtain. In the present case there is the additional difficulty that the measurements require tight vesicles in which the high-affinity sites on one side of the membrane are inaccessible to Ca^{2+} ions interacting with the low-affinity sites on the opposite site. This difficulty does not apply to study of the high-affinity sites because they are saturated at Ca^{2+} concentrations below those required for interaction with the low-affinity sites, so that accessibility of the latter is not an important factor. As a result of these problems even the simple question of whether the low-affinity sites are accessible in the unphosphorylated state E′ has not been resolved: the results of Chaloub et al.[93] and Suko et al.[94] suggest that they are accessible. Binding to E′–P under equilibrium conditions has been measured by Suko et al.,[94] but detailed data as a function of Ca^{2+} concentration were not obtained. All that can be concluded from the results is that the binding is indeed weak, with "apparent $K_{1/2}$" between 100 and 1000 M^{-1}. A similar conclusion can be reached (somewhat less rigorously) on the basis of kinetic studies that show an increase in the rate of synthesis of ATP (via the reaction $Ca_2E' - P \rightarrow Ca_2E \sim P$ in the scheme of Figure 1) as the Ca^{2+} concentration is increased.[95] It is important to note that "apparent" binding constants to E′–P have to be treated as upper limits for true equilibrium constants, because any $Ca_2E \sim P$ formed during the binding measurement would increase the apparent affinity. (In binding to the high-affinity sites the presence of E′ decreases affinity, and "apparent" constants are therefore lower limits.) It is therefore safe to conclude from these data that the association constant between Ca^{2+} and the low-affinity sites in E′–P (both sites assumed identical) must be less than 1000 M^{-1}, but it is not possible to assign a lower limit. The ability of Ca^{2+} bound to these sites to stimulate ATP synthesis sets a theoretical lower limit, which has been estimated as about 10 M^{-1}.[79]

C. How are pH Effects to be Interpreted?

Both high- and low-affinity Ca^{2+} binding is strongly affected by pH[32,95] and it is difficult to imagine that it could be pH independent. Only high-affinity binding is considered here, but the same basic principles apply to both.

There are two self-evident reasons for pH effects. One reason, especially applicable to high-affinity sites, is competition between H^+ and Ca^{2+} for the same sites. High-affinity Ca^{2+} binding sites always contain several carboxyl groups in close proximity;[9] the close proximity leads to strong electrostatic interaction between the anionic forms of these groups and anomalously high pK_a values for H^+ dissociation; as a result these groups will be partially protonated at pH values where isolated carboxyl groups would be essentially entirely

in their anionic form (see related discussion in Section II.B). The second reason is that binding studies for the Ca pump protein are carried out under conditions where protein conformational change accompanies the binding. Hydrogen ion equilibria of proteins always depend to some extent on the conformational state (the Bohr effect for hemoglobin is a prime example), and therefore, protons are always bound or dissociated when a conformational change occurs and the state of equilibrium between confomers becomes pH dependent. As noted earlier (Section IV.D), the $E \rightleftharpoons E'$ conformational change for fluorescein-conjugated pump protein has a strong pH dependence.

Proper analysis of experimental binding data requires that both effects be taken into account. For example, the data of Watanabe et al.[32] show that Ca^{2+} binding to the high-affinity sites decreases as the pH is reduced. An analysis of the results has been made that ascribes this effect entirely to direct competition,[90] and this has to be invalid. Since the unliganded protein exists predominantly in the E′ state, conversion to the E state must precede actual association of Ca^{2+} with the high-affinity sites. Data on the $E \rightleftharpoons E'$ conformational transition of the fluorescein-conjugated pump protein[81] demonstrate that a decrease in pH favors the E′ state. If a similar pH dependence applies to the unmodified native protein, it could be responsible for much of the experimentally observed decrease in Ca^{2+} binding.

Another question is whether it is useful to incorporate direct competitive effects of pH into an analysis of free-energy coupling. In the conventional thermodynamic analysis of biochemical reactions, bound or dissociated H^+ ions are not explicitly taken into account. The conventional standard free-energy of ATP hydrolysis, for example, represents the free-energy change (molar standard state) for the reaction of ATP, in its equilibrium mixture of protonation states at physiological pH, to yield ADP and Pi, each in their equilibrium mixtures of protonation states. The pK values for the compounds are such that approximately one H^+ is dissociated when ATP is hydrolyzed at physiological pH, but this is not explicitly taken into account in bioenergetic discussions, and manifests itself instead in terms of a pH dependence of the conventional $\Delta G^{o\prime}$. This conventional treatment of ATP hydrolysis has always been employed when the thermodynamics of the Ca pump reaction has been analyzed, and its use has not been questioned. It would seem logical then to apply the same principle to the incorporation of Ca^{2+} binding data into the thermodynamic analysis of the pump reaction cycle, i.e., to use pH-dependent binding constants for the association between Ca^{2+} and state E, without explicit interpretation of the pH dependence. Similarly, there is no compelling reason against applying the same principle to the $E \rightleftharpoons E'$ conformational change, i.e., to treat $\Delta G^{o\prime}$ for the isomerization as a pH-dependent parameter. There is, on the other hand, a compelling reason to separate the thermodynamics of the conformational change from the thermodynamics of binding of Ca^{2+} to state E, because the free-energy difference between the E and E′ states affects our conception of the mechanism by which free-energy transduction occurs. (In the same vein, we obviously would want to have a detailed analysis of pH effects if we intended to use the binding data as a basis for definition of the structure of the Ca^{2+} binding sites.)

Counter-transport of H^+ is a separate matter from what has been discussed in this section; it was previously considered in Section II.B. It is possible that H^+ ions (or other ions) may need to be incorporated into the kinetic transition state for a transition such as the $E \rightleftharpoons E'$ transition of Figure 1 without significant thermodynamic binding to either E or E′.

VI. ATP PROCESSING

A. What is the Structural Difference Between E ~ P and E′ – P?

The phosphorylation site of the Ca pump protein has been identified as an aspartyl group in a hydrophilic segment of the polypeptide chain. It is generally believed that the transition

from high- to low-energy phosphoenzyme ($Ca_2E \sim P \rightleftharpoons Ca_2E' - P$ in the scheme of Figure 1) does not involve displacement of the phosphoryl group from its binding site, yet the properties of the group in the two states are dramatically different, as implied by the terms "high" and "low" energy. The phosphoryl group in the E ~ P form behaves like the phosphoryl group in aspartyl phosphate and reacts rapidly with ADP to synthesize ATP. The phosphoryl group in the E' – P state is unreactive towards ADP, but is instead in reversible equilibrium with aqueous P_i. These observations imply that the standard chemical potential of the group differs by about 10 kcal/mol in the two states; similar states with a similar difference in the phosphoryl group potential occur in the reaction cycle of Na,K pumps. This raises an intriguing question of chemistry: what kind of environmental change can cause such a large change in potential? Jencks[11] has illustrated the magnitude of the problem by stating that the group in E' – P "behaves as if it were surrounded by the physically impossible concentration of 10^8 *M* carboxylate groups".

The first serious attempt to address this problem has been made by deMeis and coworkers.[8,96,97] They find that addition of organic solvents (e.g., dimethyl sulfoxide or glycerol) favors the formation of E' – P at the expense of E ~ P. They interpret this result as indicating a requirement for the dissociation of water molecules when E' – P is formed, which implies that the extraordinary stability of the E' – P complex may be due to its being in a pocket of the protein structure with a relatively nonaqueous environment. This in effect makes a concrete suggestion about the molecular changes near the substrate binding site that accompany the E → E' conformational change: the suggestion is plausible, but it rests on a single experimental observation and additional evidence is needed if it is to be substantiated.

B. What are the Equilibrium Constants for Formation of E ~ P and E' – P?

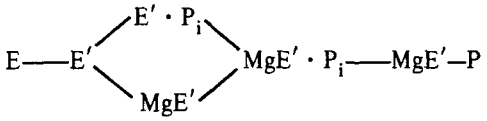
One would like to have accurate thermodynamic data for all of the successive steps involved in ATP hydrolysis: binding of ATP (including resolution of the problem discussed in Section III.B), rupture of the terminal phosphate bond of the bound ATP to form E ~ P + bound ADP, dissociation of bound ADP, conversion of E ~ P to E' – P, interconversion of E' – P and the noncovalent complex E'.P_i, and dissociation of P_i from the latter. The discussion here is limited to the two steps in which covalent phosphoenzyme intermediates are formed reversibly from the corresponding noncovalently bonded complexes. These two steps are especially interesting, though for somewhat different reasons.

The formation of E ~ P ($Ca_2E.ATP \rightleftharpoons Ca_2E \sim P.ADP$ in the scheme of Figure 1) is interesting because of its relevance to a general principle that may apply to all phosphoryl transfer enzymes.[98] The principle suggests that overall catalytic efficiency can be aided if the step in which the terminal phosphate bond of ATP is ruptured in the actual reaction cycle (i.e., in the enzyme-bound state) were flanked by states of equal free-energy, which is equivalent to saying that the equilibrium constant for this step should ideally be close to unity. There are in fact an impressive number of enzymes for which this prediction applies, i.e., the equilibrium constant for "internal" bond cleavage is close to unity regardless of the equilibrium constant for the overall catalyzed reaction, which varies from about 10^{-4} to 10^5 in the examples that have been cited. Two values of K_{eq} for the reaction $Ca_2E.ATP \rightleftharpoons Ca_2E \sim P.ADP$ have been estimated in recent reports.[30,99] The estimates (K_{eq} = 0.47, and 0.33, respectively) are in excellent agreement and provide strong support for the general principle.

It is interesting that the equilibrium constant for formation of the "low energy" phosphoenzyme intermediate is also close to unity, but in this case there is a discrepancy between the actual values for K_{eq} obtained by two procedures, one of which assumes the validity of the scheme of Figure 1 and one of which does not.

The formation of the E' – P state from unliganded protein and P_i has been studied in considerable detail.[74,100] Incorporation of one Mg^{2+} ion per P_i is required: the complex

$MgE'.P_i$ precedes formation of $MgE' - P$ and it is the equilibrium cons ization $MgE'.P_i \rightleftharpoons MgE' - P$ with which we are here concerned. If Mg^{2+} is incorporated into the scheme of Figure 1, the expanded scher $E' - P$ becomes:



In the complete absence of Ca^{2+} and ATP these are the only states of the protein that can be formed and true equilibrium data for their interconversion can be obtained experimentally by measuring the amount of phosphoenzyme as a function of P_i and Mg^{2+} concentrations. Independent data from two laboratories[74,100] are in good agreement: K_{eq} values for the isomerization reaction, pH 6.2 to pH 7, 20 to 30°C, in the absence of K^+, range from 0.6 to 1.2.

The discrepancy comes from kinetic measurements of the formation and disappearance of the $E' - P$ state under conditions similar to those used for the equilibrium measurements:[55,101] the phosphorylation rate was found to increase to values about ten times larger than the dephosphorylation rate at high concentrations of Mg^{2+} and P_i, and this means that K_{eq} for the isomerization step must have a value near ten, as compared to the value near unity obtained from the equilibrium measurements. Though the discrepancy is small, it is outside the limits of experimental error, and it therefore constitutes one of the experimental findings that raises serious questions about the validity of the scheme of Figure 1. The direct equilibrium measurements are based on this scheme and in particular assume that there is one binding site for P_i per polypeptide chain: a value of about unity for K_{eq} then results from the observation that the maximal experimental yield of $MgE' - P$ was only about 50% of the theoretical value. A value of $K_{eq} \sim 10$ would invalidate this explanation, because it would dictate that nearly all the P_i binding sites should be converted to $MgE' - P$ at saturating levels of P_i and Mg^{2+}. One would then have to conclude that 50% of the theoretical yield reflects the presence of only half the expected number of binding sites per polypeptide chain, i.e., the results would constitute strong support for an oligomeric functional unit with half-of-the-sites reactivity. (Compare the similar discussion of ATP binding sites in Section III.B.)

VII. ROLE OF PHOSPHOLIPID

A. Does Phospholipid Have a Functional Role?

The definition of active transport requires the existence of two compartments separated by a permeability barrier, which is normally a phospholipid bilayer. The question addressed here is whether there is an additional specific requirement for diacyl phospholipids at the molecular level.

Until recently it was believed by many investigators that all aspects of Ca pump function are critically dependent on retention of a lipid environment consisting minimally of an annulus of 30 phospholipid molecules.[102] Moreover, experiments done in the presence of a lipid environment indicated that fluidity of hydrocarbon chains was an essential requirement, and that hydrocarbon chain length (i.e., bilayer thickness) had a potent effect.[103,104] The view that a lipid annulus is required is no longer tenable, since pump protein dissolved in the detergent $C_{12}E_8$ (see Section III.A) with a residual phospholipid content as low as one lipid

lecule per polypeptide chain, has undiminished ATPase activity and is capable of carrying out all the important steps of the reaction cycle.[34,37] A detergent envelope around the hydrophobic (transmembrane) segment of the protein is, of course, always in a fluid state, and the detergent hydrocarbon chains have greater flexibility in how they arrange themselves than the hydrocarbon chains of a phospholipid bilayer. The greater flexibility presumably accounts for the observation of full activity in a detergent with dodecyl hydrocarbon chains, which contrasts with the considerably longer optimal chain length that is derived from experiments in which the composition of lipid is changed, but the arrangement of the lipid in the more constrained bilayer form is maintained.

On the other hand, quantitative differences between lipid-embedded and $C_{12}E_8$-solubilized pump protein certainly exist. The most important difference is that the detergent-solubilized protein is less stable, able to retain activity for only a relatively short time in a Ca^{2+}-free medium, where the protein is in the E′ state.[34,36,105] (Stability in the presence of Ca^{2+} is not significantly altered.) Quantitative differences in the binding of P_i and ATP to the E′ state have also been observed (or inferred). Some reports indicate that cooperativity in the activation of ATP hydrolysis by increasing Ca^{2+} concentration is lost,[32,60] but contrary evidence exists as well.[47,61]

Whether these differences reflect an explicit preference for association of the protein with diacyl phospholipid at one or more points of the protein surface is not clear. The pump protein is monomeric in $C_{12}E_8$ solution and altered properties could reflect the possible need of a dimeric structure for optimal stability and/or activity (see Section III.A) rather than a direct effect of the substitution of detergent for lipid. An indirect effect of $C_{12}E_8$ (i.e., via perturbation of a monomer $\rightleftharpoons$ dimer equilibrium) would not necessarily indicate a specific role of lipid in the maintenance of a dimeric structure. It could reflect a perturbation of the long octaethyleneglycol chain of $C_{12}E_8$ on the immediate environment of the parts of the protein that extend into the aqueous medium and that would not be close to lipid molecules in the native membrane.

It is clear from this discussion that it is not possible at present to exclude the possibility that specific interactions with phospholipid might make an important contribution to stability of the pump protein and/or its catalytic function, but there is also no compelling reason to believe that it does.

VIII. MECHANISM OF FREE-ENERGY TRANSDUCTION

This section discusses aspects of the transport mechanism that have been inferred on the basis of theoretical principles, experimental data, or simply intuition. The reason for the existence of these inferences is that it was for a long time difficult to imagine any conceivable mechanism that could account for active transport of any kind, so that exercise of the imagination played a useful role. The problem in this kind of situation is, of course, that not everyone draws the same inferences, so that there is now a need for the design of experiments to distinguish between the possibilities that have been suggested. Two of the questions addressed here fall into this category, the third considers an aspect of the mechanism for which even a clearly defined speculation does not exist.

A. Are Ca^{2+} and ATP Binding Sites Far Apart?

In terms of the conformational coupling principle inherent to the scheme of Figure 1, it is difficult to imagine that close contact between bound Ca^{2+} and ATP (or ADP or P_i) can be advantageous for the transport process. It seems simpler to imagine that the site at which ATP is processed is some distance from the Ca^{2+} binding sites. This would require trans-

mission of ligand binding effects over some distance through the fabric of the protein molecule, which is already a well-established phenomenon in other proteins, e.g., hemoglobin and allosteric enzymes. On the other hand, the speculative ligand conduction mechanism proposed for active transport by Mitchell[106] is dependent on close contact between P_i and the transported ion. Experiments that directly estimate the distance between the sites would clearly help to distinguish between these two radically different views of the transport mechanism.

Direct measurements of intersite distances are not available. Crude structural studies of the membrane-bound protein as a whole are relevant, and they are at least consistent with a large separation between sites because they show that the Ca pump protein in the sarcoplasmic reticulum membrane extends far into the cytoplasm.[42,107] It is reasonable to suppose that this extramembranous part of the molecule might contain the substrate processing site, whereas Ca^{2+} binding and translocation presumably involve the hydrophobic transmembrane domain of the protein. The available primary structural data discussed in relation to Section V. A support this possibility, and additional evidence of this kind is cited by Miki et al.[70]

Another approach to the problem is from the experimentally observable thermodynamic interaction between ATP and Ca^{2+} in the formation of $Ca_2E.ATP$, but the results obtained from this source are not entirely clear. Meissner,[33] more than 10 years ago, found that ATP had little effect on the association between Ca^{2+} and the high-affinity binding sites in state E, and Pickart and Jencks[30] have apparently confirmed this by showing that Ca^{2+}-free protein and Ca_2E have virtually the same association constant for ATP. Whether these data in fact support a wide separation between the binding sites is, however, unclear, because the $E \rightleftharpoons E'$ conformational change was not taken into account in these studies. Data cited earlier (Section IV.D) suggest that unliganded pump protein might exist predominantly in state E′, in which case the apparent binding constant of ATP should be different for unliganded protein and for Ca_2E even if there is no direct interaction between the sites in state E.

It is presumably safe to assume that there must be wide separation between the substrate processing sites and the Ca^{2+} sites in their low-affinity discharge state (state E′), where the latter have to be on the lumen side of the membrane. Here, too, however, the ligand conduction mechanism would predict a contrary result: this mechanism (when applied to the sarcoplasmic reticulum Ca pump) has an absolute requirement for movement of the aspartate-linked phosphoryl group across the membrane during the reaction cycle, with maintenance of close contact even in the $Ca_2E' - P$ state.[106]

B. What is the Translocation Pathway Across the Membrane?

It is reasonable to assume that the Ca pump protein possesses a transmembrane domain that resembles the transmembrane domain of bacteriorhodopsin in being "inside-out",[108] with a hydrophobic outer surface to keep the domain anchored in the bilayer and with a thread of polar amino acid side chains running through the domain to provide an incipient pathway for ion translocation. It is intuitively obvious that the incipient pathway cannot be a permanently open channel (or equivalent ionophore), nor a gated channel that is open for only part of the reaction cycle: any open passage across the membrane, simultaneously accessible from both sides, will inevitably facilitate passive downhill movement of Ca^{2+} ion, opposing active transport, regardless of the energetic factors that may be involved in creation of the passage in the course of the reaction cycle. Model calculations have been made to support this intuitive conclusion.[62] An alternating access model, as originally proposed by Jardetzky,[109] avoids this difficulty and has been generally accepted as a model for how Ca^{2+} is translocated across the membrane. In this model, the protein alternates in the reaction cycle between two conformational states (E and E′ in Figure 1), in each of which there are Ca^{2+} binding sites accessible from one side of the membrane only. If the idea of a polar thread through the transmembrane domain is accepted, then most of the polar groups

within the thread must be viewed as being tightly associated, so as to leave no room for passage of water or Ca^{2+} ions through them, and this is consistent with the properties of the polar thread in the structure of bacteriorhodopsin.[108]

The evidence presented in Section IV. A for the existence of an occluded state of the pump protein suggests a relatively trivial modification of the alternating access model for translocation, namely that access alternation involves two successive steps, with intervention of a state accessible to neither side of the membrane between the principal states E and E′.

Model calculations[62] suggest additional and more important restrictions on an alternating site model if a substantial increase in the chemical potential of the transported ion is involved, as would be true in the case of the sarcoplasmic reticulum Ca pump. The calculations illustrate the point that movement between two distinct ion binding sites (differing greatly in affinity) would occur spontaneously in a downhill direction, and would impose a barrier in the other direction that would prevent an adequate overall rate for the pump reaction. It was therefore concluded that the ion must remain bound to the same site during the change from one of the alternating conformations of the Ca pump protein to the other, i.e., the site itself must move, or the rearrangement of the polar groups of the central thread must be topologically equivalent to such movement. (The speculative structural model for Ca^{2+} translocation mentioned earlier[9] is an example of what is meant by a rearrangement "topologically equivalent" to movement of the binding site.)

This added feature of the alternating access model is still controversial. Models involving two alternating conformational states of the protein have been proposed for many other transport systems, but it is usually assumed that the transported species moves from one site to another in the course of the transport cycle; the conformational change is seen as leaving the positions of the two sites essentially fixed, while altering their relative affinities for the transported ion. The model calculations referred to above would seem to exclude this possibility for the reaction cycle of Figure 1, but it is conceivable that a more complex reaction scheme might allow it. One should, in principle, be able to answer the question experimentally. A model that involves movement of the transported ion from one site to another would require the simultaneous existence of two binding sites for each transported ion, either throughout the reaction cycle or at least in one of the reaction intermediates; in the model of the preceding paragraph there is only one binding site for each transported ion at any stage of the cycle.

It should be mentioned that there is experimental evidence (extremely weak in the opinion of this author) to suggest that both the intact Ca pump molecule and proteolytic fragments derived from it can act as an ionophore, promoting passive translocation of Ca^{2+} across the membrane,[110] and MacLennan and Klip[111] have gone so far as to construct a model for a putative transmembrane hydrophilic channel on the basis of the amino acid sequence of a proteolytic fragment that is said to have ionophoric activity. The existence of such a channel would seem to be incompatible with an alternating access model of any kind, and in particular with the reaction scheme of Figure 1. The existence of a channel per se in some part of the pump protein molecule is not necessarily incompatible with the idea of alternating access, provided that the channel does not go all the way across the membrane and is not an integral part of the translocation mechanism; it is possible, e.g., that the alternating access machinery of the protein may be several Å away from the bulk solution on one side of the membrane and that an inert aqueous channel provides a pathway for bringing Ca^{2+} ions to the alternating access site.[1] In the references cited here, however, the proposed channel or ionophore is implicitly imagined as representing the primary translocation pathway for the active transport process.

It should finally be reiterated that the ligand conduction mechanism of Mitchell[106,112] represents an extreme deviation from the principles inherent to an alternating access model for translocation. It envisages continuous access from both sides of the membrane, explicitly

assumes that the protein remains in the same conformation throughout the reaction cycle, and requires that P_i be translocated through the pump protein along with the transported ions.

C. How is Fixed Stoichiometry Assured?

Assuming a fixed stoichiometry of Ca/ATP = 2, how can we account for it in terms of a theoretical or speculative structural model? This question appears not to have been addressed at all, and the same is true for the related question for Na,K pumps. The fact that there is a problem in relation to fixed stoichiometry is readily demonstrated if the reaction scheme of Figure 1 is accepted as true. This scheme assumes that the two high-affinity binding sites for Ca^{2+} in state E are independent, which means that CaE, CaE.ATP, and CaE ~ P are possible reaction intermediates, in addition to the corresponding states containing two bound Ca^{2+} ions. If there is a really stringent restriction on stoichiometry, a place where a reaction step is physically prevented from occurring unless both Ca^{2+} binding sites are occupied, one needs to look for it elsewhere in the reaction cycle.

A logical location for such a restriction would be in the kinetic transition state for ion translocation. For example, the transition state might be constrained to be electrically neutral. Since charges derived from the protein (in the Ca^{2+} binding sites) undergo translocation along with the transported ions, a requirement for exact neutralization would fix the number of Ca^{2+} ions that need to be bound for translocation to occur. The difficulty with this explanation is that there can be no net transfer of intrinsic protein charges in the overall reaction cycle: charges translocated in one direction in the $Ca_2E \sim P \rightleftharpoons Ca_2E' - P$ transition of Figure 1 have to return in the opposite direction in the $E' \rightleftharpoons E$ transition, and the same number of charges derived from bound ions (in this case presumably H^+ and K^+) would be needed for neutralization. This would force the overall reaction to be electrically neutral, contrary to experimental fact. If the translocation step is indeed the critical point in the determination of stoichiometry, then the physical basis for it would have to be more complicated than a simple requirement for electrical neutrality.

As was previously discussed (Section IV. A), the two-conformation scheme of Figure 1 is probably not an adequate representation of the Ca pump reaction cycle. The bound Ca^{2+} ions in the state $Ca_2E \sim P$ appear to be "occluded", suggesting that the protein conformation here is not the same as in the precursor states with noncovalently bound ATP. This makes it appropriate to consider the E ~ P state (with or without noncovalently bound ADP) as a possible determinant of stoichiometry, i.e., CaE ~ P can be thermodynamically unstable relative to $Ca_2E \sim P$ even if the precursor states CaE.ATP and Ca_2E.ATP are equally stable. This explanation, however, is subject to the same difficulty as the preceding one. If a third conformational state intervenes on the pathway from E to E', then this state must presumably also intervene on the pathway from E' to E, and restrictions imposed on the composition of this state must be shown to be consistent with what is actually happening in both directions.

It is, of course, also possible that there are actually no rigid requirements anywhere in the reaction cycle. Even though CaE, CaE.ATP, and CaE ~ P may all be acceptable intermediates, their amounts may be limited sufficiently by thermodynamic and kinetic parameters to make the likelihood of translocation with only a single bound Ca^{2+} extremely low. This possibility is superficially supported by the fact that equilibrium binding of Ca^{2+} from the cytoplasmic side of the membrane is highly cooperative, which means that CaE cannot accumulate at equilibrium to a significant extent; if the Ca^{2+} binding sites and the phosphorylation site are truly independent (Section VIII. A), it would also imply that CaE.ATP and CaE ~ P would not be present at significant levels under equilibrium conditions. The stoichiometry of the overall reaction is, however, usually measured far from equilibrium, and is affected by kinetic parameters that do not influence the equilibrium in the pretranslocation steps of the cycle. One cannot assume *a priori* that there will be kinetic cooperativity

in the activation of the pump on the basis of the equilibrium binding measurements, and model calculations that could conceivably demonstrate an intimate relation between the two have not been reported. Some published results in fact suggest that kinetic cooperativity is more stringently enforced than cooperativity in the equilibrium binding process. If fixed stoichiometry in the overall reaction can be firmly established, an actual physical restriction somewhere in the reaction cycle is probably the most likely explanation. (It is worth noting that one could easily account for any fixed stoichiometry with a model involving four distinct conformational states for the pump protein. Such a model would permit two sets of paired alternating access states, each with different requirements for the kinetic transition state. There is, however, at present no experimental data that would suggest the existence of as many as four conformational states.)

IX. RELATION TO OTHER ATP-DRIVEN Ca PUMPS

The similarity between the reaction cycles of the sarcoplasmic reticulum Ca pump and plasma membrane Na,K pumps is an accepted fact. It is quite possible that there is obligatory K^+ counter-transport in the sarcoplasmic reticulum pump cycle (Section II. B), in which case the sarcoplasmic reticulum pump should perhaps be designated as a Ca,K pump; this would imply that part of the reaction cycle might be not only similar, but actually identical in the two systems. Until recently, one could argue that the kinetic similarity between the two systems might be misleading in regard to structure-function relations because Na,K pump proteins contain two different polypeptide chains, a smaller glycopeptide component of unknown function in addition to the roughly 100,000-dalton catalytic chain. Even this impediment to acceptance of similarity is now, however, questionable; a similar polypeptide is present in sarcoplasmic reticulum membranes and it has been suggested that the only difference between them may be that the glycopeptide is tightly associated with the catalytic chain in Na,K pumps, but only loosely associated in the sarcoplasmic reticulum Ca pump.[113] Evidence for participation of the glycopeptide in the sarcoplasmic reticulum Ca pump transport mechanism exists.[114]

In view of the similarity between the sarcoplasmic reticulum Ca pump and plasma membrane Na,K pumps, it is surprising that the ATP-driven Ca pump of plasma membranes appears on the basis of currently available information not to be closely related to the sarcoplasmic reticulum pump. Even more surprising are reported differences between the sarcoplasmic reticulum Ca pumps of skeletal and cardiac muscle. Are we really dealing here with different proteins (coded by different genes) for the performance of an intrinsically similar function?

A. Are Plasma Membrane Ca Pumps Intrinsically Different from the Sarcoplasmic Reticulum Ca Pump?

The extracellular Ca^{2+} concentration is about the same as the Ca^{2+} concentration in the sarcoplasmic reticulum lumen, so that plasma membrane and sarcoplasmic reticulum pumps operate against similar ion concentration gradients; both also use ATP from the same source (the cell cytoplasm). The one big difference between the two systems is that there is a membrane potential difference across the plasma membrane, of order -80 mv (inside negative) in muscle or nerve cells, whereas the potential across the sarcoplasmic reticulum membrane is zero. The electrochemical potential difference for Ca^{2+} across the plasma membrane in the resting state of a muscle cell is therefore much larger than it is across the sarcoplasmic reticulum membrane. If Equation 1 completely describes the overall reaction catalyzed by the sarcoplasmic reticulum pump, then it could not work as a pump in the plasma membrane; it would work in the opposite direction, as an ATP synthase, except at cytoplasmic Ca^{2+} concentrations in excess of 10^{-5} *M*. A different reaction cycle, with a

1:1 Ca/ATP stoichiometry would be essential for a plasma membrane protein intended to help maintain intracellular Ca^{2+} at a very low level. If, on the other hand, Equation 1 is incomplete and obligatory counter-transport of other cations is part of the pump cycle, then the effect of the membrane potential would be reduced; if the pumps were not electrogenic at all (an untenable supposition; see Section II. B) the membrane potential would have no effect; if there is net transfer of one positive charge per cycle (e.g., counter-transport of three H^+) an unmodified sarcoplasmic reticulum pump would work as a pump in the plasma membrane down to a cytoplasmic Ca^{2+} concentration of close to 10^{-7} *M*. The question of whether we can theoretically even entertain the idea that the same pump protein might be used in plasma and sarcoplasmic reticulum membranes therefore depends on the answer to the question posed by Section II. B, which we do not yet have.

The experimental evidence[115-117] indicates that the plasma membrane protein differs significantly from the sarcoplasmic reticulum protein. The catalytic polypeptide chain has a higher molecular weight and a substantially different amino acid composition. The stoichiometry of the overall reaction (Ca/ATP = 1 or 2) and the related question of electrogenicity are still unresolved: a claim that the overall process is an electroneutral Ca^{2+}/H^+ exchange[118] has been questioned by the same laboratory that made it.[115] The reaction cycle, apart from the question of stoichiometry, is intrinsically similar to that given for the sarcoplasmic reticulum pump in Figure 1, but there is a major functional difference in that the plasma membrane pump is stimulated by calmodulin, whereas calmodulin almost certainly has no effect on the skeletal muscle sarcoplasmic reticulum pump,[114] though it may have an indirect effect in cardiac sarcoplasmic reticulum (see below). Most of the data for the plasma membrane pump come from experiments with the protein from the red blood cell, but it is probable that plasma membrane pumps from other cells, e.g., from sarcolemma, are identical or essentially similar.

B. Does the Cardiac Sarcoplasmic Reticulum Ca Pump Differ from the Skeletal Muscle Pump?

The cardiac sarcoplasmic reticulum Ca pump differs physiologically from the skeletal muscle pump in that it is stimulated by catecholamines. This effect is ascribed to a small protein called phospholamban (molecular weight 22,000), which is present in cardiac sarcoplasmic reticulum membranes, but has not been detected in skeletal muscle membranes.[119-123] Phospholamban is phosphorylated by a cAMP-dependent protein kinase, also a constituent of the cardiac sarcoplasmic reticulum membrane, and it is the phosphorylated form of the protein that stimulates pump activity. A calmodulin-dependent kinase provides an additional pathway for phosphorylation.

Does this well-established phenomenon indicate an intrinsic difference between the cardiac and skeletal muscle pump proteins? Or can it be ascribed entirely to the presence of phospholamban in one membrane and not in the other, the pump proteins themselves being identical? These questions have not been resolved. It has been reported that skeletal muscle sarcoplasmic reticulum has a cAMP-dependent kinase, which introduces an alkyl (rather than an acyl) phosphate group either into the pump protein itself or into another protein of similar molecular weight, and that pump activity is stimulated thereby.[124,125] It has also been reported that the glycopeptide of skeletal muscle membranes (a possible analog of the glycopeptide of Na,K pumps) can be phosphorylated by a calmodulin-dependent protein kinase with resulting stimulation of the pump.[114] These observations are not really relevant to the question of whether or not the cardiac and skeletal muscle pumps are different, because the proteins said to be phosphorylated in skeletal muscle sarcoplasmic reticulum are not the same as the protein that is phosphorylated in cardiac sarcoplasmic reticulum. They do, however, raise new questions about the skeletal muscle pump and its reaction mechanism: if alkyl phosphorylation of the pump protein or of noncatalytic polypeptides is indeed

functionally important, then one needs to ask what steps of the reaction cycle are affected, what thermodynamic or kinetic parameters are involved, etc.

X. CONCLUDING REMARKS

Some time after the manuscript for this review was completed, a new paper from Inesi's laboratory[126] retracted previous work on the subject of site stoichiometry and came to the conclusion that there are two high-affinity Ca^{2+} binding sites and one high-affinity ATP binding site per polypeptide chain. Moreover, close to stoichiometric conversion of bound ATP to phosphoenzyme was observed. These new results are consistent with the minimal reaction cycle of Figure 1 and remove the principal evidence that might have supported a mechanism based on half-of-the-sites reactivity (see Section III. B). A major experimental innovation in the new study was the use of molecular sieve HPLC for separation and quantitation of solubilized protein, which permitted measurement of incorporation of radioactive Ca^{2+} or phosphate at a higher level of purity of the Ca pump protein. Previously reported relatively low yields of radioactivity (per gram of protein) were clearly affected by the presence of contaminating proteins and/or Ca pump protein that had been denatured. Another study suggesting a similar explanation for low yields of phosphoenzyme is that of Pickart and Jencks,[29,30] cited earlier in the text.

This new study may represent a final resolution of Sections III. B and C of this review. What is perhaps more important in relation to all of the questions in this review is that the new study involves correction of data previously reported from the same laboratory. This is not intended as a criticism. Everyone who has worked with membrane proteins knows that they are more difficult to purify and characterize than crystallizable water-soluble enzymes. It does, however, challenge a generally accepted dogma about the nature of scientific progress. The dogma visualizes an ideal world, in which experimental results are permanent truths, but theory and hypothesis are short-lived: a single experimental inconsistency is considered sufficient to eliminate a theoretical or hypothetical mechanism. In the real world of membrane transport this principle clearly does not apply: experiment and theory must both be considered as fluid, and when there is a conflict between them, it is not necessarily the latter that must give way.

With this in mind, I should like to hazard the conclusion that the questions raised in this paper do not constitute *compelling* reasons for discarding the principal features of the transport mechanism implicit to the reaction cycle of Figure 1:

1. The transport protein is functionally monomeric and alternates between two conformational states.
2. One state contains the free-energy donating substrate at a high chemical potential and the transported ion at a low chemical potential; the second state contains the substrate at a low potential and the transported ion at a high potential.
3. The binding site for the transported ion is accessible only from the cytoplasm in one state and only from the opposite side of the membrane in the other state.
4. Translocation of the transported ion and free-energy exchange with the substrate occur in synchrony in a single step, linked to the transition of the protein from one conformational state to the other.

The questions raised in the review have shown that the experimental support for the model is flimsy at best, and they suggest that some minor revisions will certainly be needed. However, as regards to the experimental data that dispute the basic features of the model per se (e.g., Section IV. B), it is too early to decide whether they will ultimately require an entirely new model, based on fundamentally different principles, or whether (as in the case

of the equally serious questions based on stoichiometry) it is the data that will prove to need revision.

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