Review Letter

The sarcoplasmic reticulum calcium pump

Localization of free energy transfer to discrete steps of the reaction cycle

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Free energy transduction in active transport resembles other protein-catalyzed processes, occurring by an ordered sequence of discrete bond-breaking and bond-making steps. The bonds that affect the transported ion directly are chelation bonds, which alter the chemical potential of the bound ion, but not its chemical identity. Available data for the sarcoplasmic reticulum Ca pump (admittedly incomplete) suggest that more than 50% of the free energy transfer may be localized to a single step of the reaction cycle.

Active transport *Calcium pump* *Conformational change* *Free energy transduction*

1. INTRODUCTION

Hill and Eisenberg [1] have asked the question: 'Can free energy transduction be localized at some crucial part of the enzymatic cycle?' This paper addresses this question with reference to experimental data for the sarcoplasmic reticulum (SR) Ca pump.

It can be argued that the question raised by Hill and Eisenberg is a secondary question, and that the answer depends on a more basic question: 'Do active transport proteins catalyze rigidly coupled reactions, tailored to the task they must perform?' Hexokinase is a familiar example of what is meant [2]. It catalyzes the reversible formation of glucose 6-phosphate from ATP and glucose. Isomeric products are not formed, there is no transfer of a second phosphoryl group to the initial product, no catalysis of the uncoupled hydrolysis of glucose 6-phosphate, and no significant catalysis of uncoupled ATP hydrolysis. Are active transport proteins similarly dedicated to a single purpose, with all side reactions virtually excluded? If so, then the transport cycle must consist of an ordered sequence of well defined steps, and, as has been done for hexokinase, chemical and physical methods can be used to investigate exactly what happens (energetically as well as chemically) in each step. If not, then the analysis becomes more complicated.

Hill [1,3] has consistently discussed the theory of active transport in terms of models requiring complex analysis, and this may be the reason why his conclusion in regard to localization of free energy transduction differs from the conclusion of this paper. Fig.1a represents Hill's model for a simplified ATP-driven pump: in the original model one Na^+ and one K^+ are transported uphill in opposite directions, and the model is here adapted to the uphill transport of two Ca^{2+} in the same direction. Fig.1b shows for comparison the conventional 'minimal' model of DeMeis for the SR Ca pump [4], which is based on experimental data. Hill's model resembles the conventional model in having two alternating conformational states (here designated E and E′), in which binding sites for the transported ion are accessible from opposite sides of the membrane. It also incorporates the experimentally established difference between the

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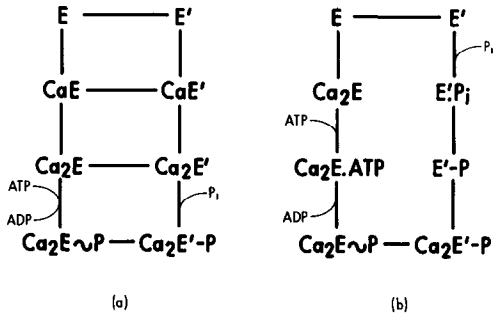

Fig.1. (a) The active transport model of Hill, adapted from figs.2.6 and 2.7 of [3]. (b) A skeletal form of the DeMeis reaction cycle for the SR Ca pump [4]. In both schemes, Ca^{2+} binds from the cytoplasmic side of the membrane in state E and from the SR lumen side in state E'.

chemical properties of the covalently bound phosphoryl group in the same two states (here represented by the use of different symbols for the covalent bond). However, Hill's model differs from the DeMeis' model in that Hill explicitly assumes that the interconversion between the E and E' states of fig.1 can occur in any binding state of the protein, whereas the DeMeis model permits interconversion only in the unliganded state ($E \rightleftharpoons E'$) and in the state in which all ligand binding sites are occupied ($Ca_2E{\sim}P \rightleftharpoons Ca_2E'$-P). Removal of the latter restriction leads to the possibility of several different reaction cycles with different reactants and products [3]. To be specific, fig.1a allows for 6 different cycles: one cycle catalyzes coupled transport with a stoichiometry of Ca/ATP = 2, one cycle catalyzes coupled transport with Ca/ATP = 1, one cycle catalyzes uncoupled ATP hydrolysis, and 3 cycles facilitate uncoupled passive downhill movement of Ca^{2+}. In this model the overall reaction stoichiometry is not fixed, but depends on the relative rates of the different possible cycles, which would in turn be a function of external conditions. There is no unique equilibrium state, but instead a steady state sensitive to kinetic parameters and environmental conditions; free energy transduction becomes critically dependent on kinetic as well as thermodynamic parameters; analysis requires the elegant methods that Hill has devised for dealing with multicycle kinetics [3].

The situation is quite different for the DeMeis model in fig.1b. This is a single cycle sequence, with precisely defined successive steps, catalyzing only the single reaction

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

where 'cyto' and 'SR' refer respectively to the cytoplasmic and SR lumen sides of the membrane. The transport stoichiometry is fixed at Ca/ATP = 2, independent of the state of the system, and eq.1 has a uniquely defined equilibrium state [5], as does any chemical reaction of fixed stoichiometry. The problem of determining how free energy is transferred from ATP to the transported Ca^{2+} should for this model be operationally similar to the problem of determining how a phosphate group is transferred from ATP to glucose by the action of hexokinase. The model of fig.1b is a 'minimal' model, and many details are still unresolved, but the fixed stoichiometry, and therefore the principle of an ordered reaction sequence, are supported by a large body of experimental data [5,6].

It should be noted in this connection that transformation of one kind of energy into another is by definition a thermodynamic phenomenon, and that free energy transduction in active transport needs to be defined in terms of thermodynamic parameters. This creates no difficulty for a reaction with fixed stoichiometry because free energy dissipation in such a reaction occurs only when the system is not at equilibrium, and the extent of deviation from the equilibrium state then quantitatively determines free energy loss per reaction cycle. Free energy transfer parameters per se are determined by the parameters of the equilibrium state: equilibrium constants for individual steps of the reaction cycle and for related reactions [7].

2. CONFORMATIONAL STATES AND THE FREE ENERGY DIFFERENCE BETWEEN THEM

Conformational transitions play a crucial role in active transport, and it is therefore necessary to have operational definitions of the terms 'conformational state' and 'conformational change'. No generally accepted rules appear to exist; the following considerations provide definitions for the purpose of this paper.

(i) It has to be recognized that a protein molecule in a given state does not have a rigidly fixed structure, but fluctuates between numerous micro-states of about equal free energy: these fluctuations are here regarded as attributes of any definable single state and changes in the distribution between different micro-states (e.g., as a function of pH) are not treated as a conformational change.

(ii) It is hardly conceivable that binding of a ligand to a pre-existing binding site in a particular state 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.

(iii) Changes in long-range order or changes in chemical accessibility (e.g., SH groups or peptide groups subject to proteolysis) can provide unambiguous evidence for conformational change. Changes in spectral properties (most commonly fluorescence) are usually considered as indicative of conformational change, and this convention is followed here though this is not entirely safe: it is possible that binding of a ligand to a fixed site can affect spectral parameters even in the absence of structural change [8].

(iv) Existence of more than one conformational state can sometimes be inferred on the basis of cooperativity in binding or kinetics, as in allosteric enzymes [9].

The free energy difference between conformational states is an essential parameter for thermodynamic analysis, but its experimental measurement may not be easy. Transition from one conformation to another is often induced by the binding of a ligand with high affinity for the second conformation: ability to do this is essentially definitive for use of the term 'allosteric'. The value of ΔG° derived from the equilibrium constant for such an induced transition would be the sum of two free energies, one for the conformational change itself and one for the binding of ligand, the latter reflecting the difference in binding affinity between the two conformations. This combined ΔG° is not helpful for mechanistic analysis; separate values for the component contributions are needed for that; one needs to be able to measure the equilibrium constant for the conformational transition in the absence of ligand, or to extrapolate data obtained in the presence of ligand to that point, or to obtain the equivalent information from rate constants measured in kinetic experiments.

The following analysis will assume that the SR Ca pump protein has only the two conformational states E and E′ shown in the skeletal schemes of fig.1. It is likely that this assumption may be incorrect, but the present state of knowledge about the reaction cycle as a whole does not justify an attempt to incorporate possible additional states into the analysis. (See section 5 for further comments.)

Making the same assumption, that E and E′ represent the only conformational states of the protein, Pick and Karlish [10] have properly determined the equilibrium constant for the transition between them, but they used an inactive fluorescein-conjugated form of the protein and not the native protein, and it is clear from their measured dependence of the equilibrium on Ca^{2+} concentration that conjugation has affected their result ($\Delta G^\circ = 0$ at pH 7 and room temperature). The normally observed cooperativity for Ca_2E formation [11] in fact requires (when only two conformational states are assumed) that the E′/E equilibrium constant must be >100 under these conditions [12]. A value of K_{eq} higher than about 10^4 is excluded for other reasons. I shall hereafter use $\Delta G^\circ = -3$ kcal/mol for the E $\rightleftharpoons$ E′ transition, which corresponds to $K_{eq} = 150$.

3. CHEMICAL POTENTIALS OF BOUND LIGANDS

In order to obtain quantitative information about the transfer of free energy from a donor such as ATP to a transported ion, it is clearly necessary to use separate thermodynamic parameters for donor and acceptor species in their protein-bound states, and to follow the changes in these parameters as the protein goes through its reaction cycle. Thermodynamics being a very flexible discipline, there are different possible pro-

cedures for doing this, e.g., Jencks [13] has used 'binding energies' as the basic parameters. I have suggested chemical potentials of the bound species as particularly well suited to the problem [14]. Standard potentials have proved to be the most useful form of this parameter: for bound phosphate groups they are closely related to the 'group potentials' introduced many years ago by Lipmann [15]; for noncovalently bound species they are closely related to standard free energies of ligand binding [14]. Relative standard potentials for a bound ligand i ($\mu^{o'}_{i,b} - \mu^{o'}_{i,aq}$) are unambiguous measures of the escaping tendency of the ligand from the bound state. (The prime here and throughout this paper indicates the normal biochemical standard state: pH ~7, with Mg^{2+} at an appropriate concentration treated as a solvent component even where it is actually known to be a direct participant in a reaction.) Hill and Eisenberg [1], and subsequently Hill [16], have objected to the use of standard chemical potentials for analysis of free energy transduction, but it is difficult to understand the basis for the objection. Hill [16] has stated that "the free energy of the system at intermediate stages of the cycle is inextricably mixed up among the enzyme and the two ligands and cannot be assigned to the separate ligands." This is true, but it is equally true for liquid solutions where the solvent is an intimate participant in all reactions. Chemical potentials were introduced into solution thermodynamics by Gibbs to cope with this problem. Local interactions make it invalid to equate the total free energy in a system with the sum of the molar free energies of the components, but it follows from the definition of μ_i that it is always valid to set $G = \Sigma n_i \mu_i$

4. THE $Ca_2E{\sim}P \rightleftharpoons Ca_2E'{-}P$ TRANSITION

It has been surmised for a long time that the transition between $Ca_2E{\sim}P$ and $Ca_2E'{-}P$ is likely to be the critical free energy coupling step in the SR pump mechanism. This is the step in which protein-bound Ca^{2+} is translocated to the SR lumen side of the membrane. The binding constant for Ca^{2+} must decrease drastically when this happens, in order to allow the bound Ca^{2+} to be released into the luminal space, where the free Ca^{2+} concentration is normally > 1 mM. (Rapid release following the translocation step is experimentally established.) A change in chemical reactivity of the bound phosphoryl group, from 'high energy' E~P to 'low energy' E′–P is also synchronous with translocation; the core of the chemi-osmotic exchange process appears to be localized in this single step. Makinose [17] was perhaps the first to propose this, but the most direct evidence comes from operation of the pump in the reverse direction (ATP synthesis induced by a high luminal Ca^{2+} concentration). There is an excellent summary of the data by DeMeis and Vianna [4].

A definitive value of ΔG^o for the $Ca_2E{\sim}P \rightleftharpoons Ca_2E'{-}P$ transition does not exist. Suko et al. [18] have shown that intravesicular Ca^{2+} increases the equilibrium level of total phosphoenzyme formed from E′ and P_i in sarcoplasmic reticulum vesicles in the absence of extravesicular Ca^{2+}, and this result can be used to obtain an estimate. I assume again that E and E′ are the only existing conformational states. Since Ca^{2+} binding and phosphorylation occur on opposite sides of the membrane, it becomes reasonable to assume that the binding affinity of E′ for intravesicular Ca^{2+} should be unaffected by phosphorylation, from which it follows that Ca^{2+} should not affect the equilibrium level of phosphorylation in the E′ state. The observed change in total phosphoenzyme (about 3-fold) must then be ascribed to formation of $Ca_2E{\sim}P$: an equilibrium constant $[Ca_2E{\sim}P]/[Ca_2E'{-}P] = K_{eq} \sim 10$ is required. (Suko et al. do not allow for the possibility of a conformational transition, and interpret their results in a different way. The interpretation given here may not be consistent with their data on the effects of Mg^{2+} and P_i on the yield of phosphoenzyme.) Another way to get a value for K_{eq} is from the equilibrium constant of 4.7×10^5 M^{-2} given by Pickart and Jencks [19] for the combined reaction, $2Ca^{2+} + E'{-}P \rightleftharpoons Ca_2E{\sim}P$. Assuming a binding constant between 10 and 1000 M^{-1} for binding of Ca^{2+} to the SR-facing sites on E′ [20], one obtains a range of 0.5–5000 for K_{eq}. The value deduced from the data of Suko et al. is in the middle of this range and will be used for the following calculations. It corresponds to $\Delta G^{o'} = +1.4$ kcal/mol for the transition $Ca_2E{\sim}P \rightleftharpoons Ca_2E'{-}P$, and to a Ca^{2+} binding constant of 200 M^{-1}. An earlier estimate for the Ca^{2+} binding constant [11], based on computer fit of experimental data, is 300 M^{-1}.

Completion of the analysis requires knowledge of the standard chemical potentials of the bound ligands in $Ca_2E{\sim}P$ and $Ca_2E'{-}P$. The potentials are intrinsically determined by the chelating groups in the binding sites [21] and could in principle be estimated without thermodynamic data at all if sufficient structural information were available. Here I rely on binding data, i.e., equilibrium constants for the reactions, protein + aqueous ligand = = complex. These data can be used to evaluate $\mu^{o'}_{l,b} - \mu^{o'}_{l,aq}$ if the binding process occurs without change in protein conformation and without change in the thermodynamic state of other ligands that may be present. Ultimate proof that the second condition is satisfied may require direct demonstration that no structural change in the binding has occurred; at the present time inferences similar to those used in the preceding paragraph have to suffice.

The estimates made here are based on recent data of Pickart and Jencks [19]; independent results from other laboratories are in good agreement. The data are for 25°C, pH 7, 5 mM Mg^{2+}, 100 mM K^+. Mg^{2+} and K^+ are treated as solvent components. The data do not explicitly distinguish between different conformational states of the pump protein. In accord with the E'/E equilibrium constant given above, it is appropriate to consider unliganded protein as being entirely in the E' state. The assignment of unspecified 'E' to conformations E or E' is self-evident for all other cycle intermediates used by Pickart and Jencks, if it is assumed that only these two conformations exist. The data may then be summarized as follows:

$$E' + 2Ca^{2+} \rightleftharpoons Ca_2E \qquad \Delta G^{o'}_1 = -15.3 \text{ kcal/mol}$$

$$Ca_2E + ATP \rightleftharpoons Ca_2E \cdot ATP \qquad \Delta G^{o'}_2 = -6.6 \text{ kcal/mol}$$

$$Ca_2E \cdot ATP \rightleftharpoons Ca_2E{\sim}P \cdot ADP \qquad \Delta G^{o'}_3 = +0.4 \text{ kcal/mol}$$

$$Ca_2E{\sim}P \cdot ADP \rightleftharpoons Ca_2E{\sim}P + ADP \qquad \Delta G^{o'}_4 = +4.3 \text{ kcal/mol}$$

$$E' + P_i \rightleftharpoons E' \cdot P_i \qquad \Delta G^{o'}_5 = -1.9 \text{ kcal/mol}$$

$$E' \cdot P_i \rightleftharpoons E'{-}P \qquad \Delta G^{o'}_6 = -0.3 \text{ kcal/mol}$$

The standard chemical potentials of bound ligands obtained from these data and from data cited earlier in the text are given in table 1. The calculations for $Ca_2E'{-}P$ are straightforward: $\mu^{o'}_{a,b}$ is obtained from the binding constant of 200 M^{-1} given earlier; $\mu^{o'}_{P,b}$ is the sum of $\Delta G^{o'}_5$ and $\Delta G^{o'}_6$. The calculations for $Ca_2E{\sim}P$ are less simple because it has not been experimentally possible to measure the reversible dissociation of Ca^{2+} from this intermediate with the E~P bond intact (see section 5). Ca^{2+} does, however, dissociate reversibly from Ca_2E, and $\mu^{o'}_{a,b}$ for this state is therefore calculable from $\Delta G^{o'}_1$ and $\Delta G^{o'}$ for the $E' \rightleftharpoons E$ conformational change. I have assumed that what happens at the phosphorylation site has no effect on $\mu^{o'}_{a,b}$ as long as the protein remains in the E conformation, so that the value calculated for Ca_2E is used for $Ca_2E{\sim}P$ as well. With this assumption one can calculate $\mu^{o'}_{P,b}$ by subtracting $\Delta G^{o'}$ for ATP hydrolysis (−7.5 kcal/mol) from $\Delta G^{o'}_2 + \Delta G^{o'}_3 + \Delta G^{o'}_4$. In the absence of protein conformational change and with $\mu^{o'}_{a,b}$ constant, these free energies exclusively reflect thermodynamic changes arising from the successive chemical changes of bound ATP.

Table 1

Thermodynamic data for the $Ca_2E{\sim}P \rightleftharpoons Ca_2E'{-}P$ transition

	$Ca_2E{\sim}P$	$Ca_2E'{-}P$
$\mu^{o'}_{a,b} - \mu^{o'}_{a,aq}$ (kcal/mol)	−9.2	−3.2
$\mu^{o'}_{P,b} - \mu^{o'}_{P,aq}$ (kcal/mol)	+5.6	−2.2

Data for the transition	
$2 \times \Delta\mu^{o'}_{a,b}$	+12.0 kcal/mol
$\Delta\mu^{o'}_{P,b}$	− 7.8 kcal/mol
$\Delta\mu^{o'}_{protein}$	− 3.0 kcal/mol
Total $\Delta G^{o'}$	+ 1.2 kcal/mol

The chemical potential changes for the transition from $Ca_2E{\sim}P$ to $Ca_2E'{-}P$ are also shown in the table. The protein undergoes a conformational change in this transition, and the free energy change for this structural alteration must be added to the changes in ligand potential to arrive at the total $\Delta G^{o'}$ for the transition. The value of −3 kcal/mol was estimated earlier in the text. The sum of this value and the $\Delta\mu^{o'}$ values for the bound ligands is seen to be essentially identical to the overall $\Delta G^{o'}$ estimated earlier for the transition, which shows that all the calculations made here are self-consistent.

5. DISCUSSION

The results in table 1 provide strong support for the contention that most of the free energy transfer from ATP to the transported ions occurs in the SR Ca pump cycle in a single step of the cycle, in the transition between $Ca_2E{\sim}P$ and $Ca_2E'{-}P$. The maximal free energy that can be transferred in the overall reaction (at equilibrium, in the absence of free energy dissipation) has been estimated as 13.85 kcal/mol [7]: the Ca^{2+} clearly gains nearly all of this free energy in this single step, and the loss in the potential of the bound phosphoryl group corresponds to more than half the free energy derived from ATP hydrolysis. It is significant that the changes in ligand free energy are seen at the level of the standard potential, which means that they reflect changes in intrinsic binding affinity, presumably caused by movement of chelating groups in and out of the binding sites. It is also significant that the $\Delta\mu^{o'}$ values for the ligands are numerically much larger than $\Delta\mu^{o'}$ for the protein conformational change. This means that we are dealing largely with direct transfer of free energy from one ligand to the other [14], by means of thermodynamically compensatory structural changes at the binding sites: the protein provides the structural link between the changes at the binding sites, but plays only a minor role as an energetic participant. The protein is, however, not entirely energetically inert and the data demonstrate a measure of applicability of the ideas inherent in the 'rack' mechanism of Lumry [22], though it involves only a fraction of the total free energy. (The E state is less stable than the E′ state by 3 kcal/mol, but a high binding constant for ATP helps to overcome this and to prevent the protein from being locked into the E′ conformation. The conformational free energy is recovered in the $Ca_2E{\sim}P \rightleftharpoons Ca_2E'{-}P$ transition and helps to reduce the total free energy difference between the two states.)

These conclusions about the free energy transduction process should not be taken seriously and they are not being offered as a 'solution' to the free energy transduction problem. There are still many uncertainties at the experimental level, as illustrated by the many assumptions that had to be made to arrive at the results of table 1. Most questionable is the assumption that E and E′ are the only two conformational states through which the pump protein passes in the course of each reaction cycle. As has already been noted, Ca^{2+} in the state $Ca_2E{\sim}P$ is not as readily accessible to the cytoplasmic solution as it is in the states Ca_2E and $Ca_2E \cdot ATP$, which suggests that the site translocation that defines states E and E′ may proceed via an intermediate 'occluded' state in which the Ca^{2+} binding sites are accessible from neither side of the membrane [23,24]. (If so, the same intermediate would presumably occur on the pathway between unliganded E and E′.) This and other questions about the reaction cycle will, of course, eventually be resolved. A complete understanding of the mechanism may require more than this and may not be achieved until after the three-dimensional structure of the pump protein is known. This kind of reservation is not unique to transport proteins: knowledge of the three-dimensional structure has proved essential, for example, for a full understanding of the mechanism of action of hexokinase [25].

These reservations are unimportant in light of the purpose of this paper, which has been to address the question raised by Hill and Eisenberg [1]. The paper demonstrates that catalysis of free energy transfer in active transport can be analyzed step-by-step in much the same way as is done for enzymic catalysis of biochemical transformations. Even what happens in individual steps is probably similar for the two kinds of catalysis, because the only way a protein can affect a bound ligand (whatever the ultimate purpose) is by breaking and making of covalent or non-covalent chemical bonds: discrete events in a reaction cycle that can always be localized to particular steps. Catalysis of free energy transduction does not occur as if by magic, as a result of fortuitous relations between rate constants; it occurs instead by an ordered sequence of breaking of old bonds and making of new bonds. The unique feature in the mechanism of active transport is that the bonds that affect the transported ion are chelation bonds which alter the chemical potential of the bound ion but do not alter its chemical identity.

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