Equilibrium Constants for Some Steps of the Reaction Cycle of the Sarcoplasmic Reticulum Calcium Pump*

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This paper summarizes true equilibrium measurements for some partial reactions of the sarcoplasmic reticulum calcium pump transport cycle. The most important result is the estimation of the equilibrium constant for the interconversion of the two major conformational states of the protein, E (Ca^{2+} binding sites facing the cytoplasm) and E′ (Ca^{2+} binding sides facing the sarcoplasmic reticulum lumen). The value of $K_0 = [E']/[E]$ cannot be evaluated directly by any method available at present, but observed cooperativity in the binding of Mg^{2+} and Ca^{2+} to unliganded protein strongly indictes that $K_0 \gg 1$. The most probable value, valid within an order of magnitude, is $K_0 \simeq 10^3$, *i.e.*, the E′ state is more stable than the E state by about 4 kcal/mol.

Introduction

It is now generally accepted by many workers in the field [1, 2] that the minimal reaction mechanism of the sarcoplasmic reticulum calcium pump involves alternation between two distinct conformational states, here designated as E and E′. In E, the Ca^{2+} binding sites face the cytoplasm and they have high affinity for Ca^{2+}. In E′ the Ca^{2+} binding sites face the sarcoplasmic reticulum lumen and they have low affinity for Ca^{2+}. E has binding sites for ATP and Mg^{2+}, and, when both these sites and the Ca^{2+} sites are filled, can be converted to the high energy phosphoenzyme derivative $E \sim P$. E′ has binding sites for P_i and Mg^{2+}, and can be converted to the low energy phosphoenzyme derivative $E' - P$. This minimal mechanism and the accompanying properties of the E and E′ states are basic assumptions on which the analysis of this paper is based. The results obtained could be subject to reinterpretation if the pump mechanism were ultimately to be shown to have important features that the minimal mechanism does not take into account.

Some of the partial reactions of the complete pump cycle can be studied in the laboratory under conditions where completion of the cycle cannot occur (*i.e.*, absence of ATP and/or Ca^{2+}), and under these conditions true thermodynamic equilibrium data for the partial reactions can be obtained. This paper summarizes results of this kind, taken mostly from work in our own laboratory [3, 4]. In addition, it offers a new interpretation of data of Inesi *et al.* [5] for the binding of Ca^{2+} to the high affinity sites of E. Self-consistent values for the equilibrium constant of the $E \rightleftharpoons E'$ interconversion are obtained from both these sources. This equilibrium constant provides us with the free energy difference between the two conformational states of the protein, an important parameter for understanding of the intrinsic thermodynamic pathway of free energy transduction by the pump protein.

Methods

Experimental data summarized here are based on measurement of the extent of phosphorylation of the E′ form as a function of the concentrations of P_i and Mg^{2+} and as a function of temperature. The protein was membrane-bound, in leaky vesicles, and measurements were made at pH 6.2, in the absence of added KCl, and in the presence of sufficient EGTA to suppress the level of free Ca^{2+} to insignificantly low levels. A detailed description has been published [3, 4]. The experimental method is essentially the same as that previously employed by Punzengruber *et al.* [6].

Summary of Results

As is well established, Mg^{2+} is required for phosphorylation by P_i and the final product has the formula $MgE' - P$. If measurements are made at increasing free P_i concentration, $[P_i]$, but at constant

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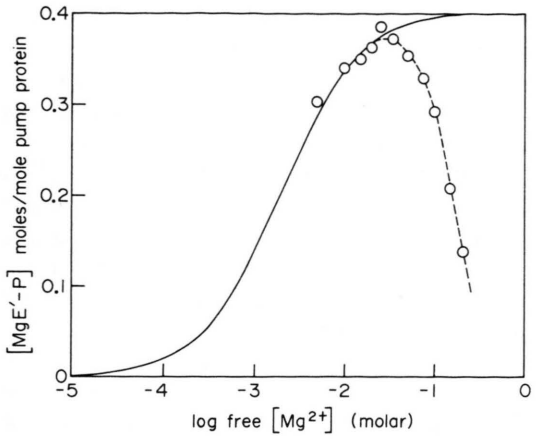

Fig. 1. Typical curve for the yield of phosphoenzyme as a function of the *free* Mg^{2+} concentration, at constant *free* P_i concentration (5 mM). The solid curve is a calculated curve, based on values for K_1 to K_5 derived from parallel studies at lower Mg^{2+} concentrations.

$[Mg^{2+}]$, the yield of $[MgE'-P]$ is found to increase as expected on the basis of 1 : 1 stoichiometry, and to approach saturation at high $[P_i]$. On the other hand, if $[Mg^{2+}]$ is varied at constant $[P_i]$, the yield of $[MgE'-P]$ increases only at first, and then drops sharply at $[Mg^{2+}] > 30$ mM, as shown in Fig. 1. Dilution from the highest Mg^{2+} concentration employed reversed the decrease in $[MgE'-P]$, demonstrating that all the results obtained represented thermodynamic equilibrium data [4].

Results obtained at relatively low $[Mg^{2+}]$ at 20 °C were in excellent agreement with similar data of Punzengruber *et al.* [6]. Dependence of $[MgE'-P]$ on the concentrations of Mg^{2+} and P_i could be quantitatively accounted for in terms of the right-hand portion of the scheme of Fig. 2. Analysis of these data alone could not, however, yield unambiguous equilibrium constants because the experimental measurements and the parameters describing their dependence on $[Mg^{2+}]$ and $[P_i]$ do not permit a distinction between species that differ in the conformational state of the protein, but otherwise have the same composition. The first phosphate binding constant obtained from the data, for example, is formally equal to

$$\frac{[E'\cdot P_i] + [E\cdot P_i]}{([E'] + [E])[P_i]} = \frac{K_9/K_0 + K_1}{(1 + 1/K_0)}. \qquad [1]$$

The same limitation applies to the similar analyses of Punzengruber *et al.* [6] and Epstein *et al.* [7]. It is only the demonstration that $K_0 \gg 1$ (see below), from which it follows that the E species in Eqn. (1) must be present at much lower concentrations than the corresponding E′ species, that permits identification of the experimental equilibrium quotient with $[E'\cdot P_i]/[E'][P_i] = K_1$. The values for K_1 to K_5 listed in Table I are all dependent in this manner on the knowledge that $K_0 \gg 1$.

This part of our work was done at several temperatures and the temperature dependence of K_1 to K_5 was used to determine the corresponding enthalpies, ΔH_1 to ΔH_5, which are listed in Table II. It is seen that the ΔH values we obtained do not agree with the extraordinarily high ΔH values reported for the same reactions by Epstein *et al.* [7]. This is an important result because Racker [8] used the high $\Delta \bar{H}$ values as the basis for a novel (but inherently implausible) theory of transport energetics. The ΔH values of Epstein *et al.* were derived from calorimetic measurements, and a likely reason why these measurements might have yielded data that could not be interpreted as arising from the reactions in Fig. 2 has been suggested [3].

Analysis of the inhibitory effect of high $[Mg^{2+}]$ on phosphoenzyme formation showed that precisely $3Mg^{2+}$ ions must be able to bind to the unphosphory-

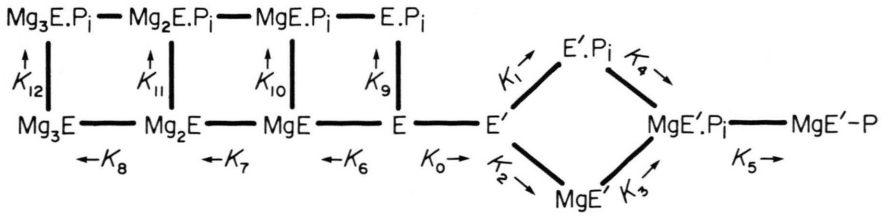

Fig. 2. Minimal reaction scheme required to account for the experimental results. The arrows indicate the directions of each reaction step used for definition of the corresponding K, *e.g.*, $K_0 = [E']/[E]$, $K_6 = [MgE]/[E][Mg^{2+}]$, $K_9 = [E\cdot P_i]/[E][P_i]$. The equilibrium constants relating the P_i-containing forms of E are not independent, so that no symbols for them are required.

Table I. Summary of equilibrium constants[a].

$E \rightleftharpoons E'$	K_0	(1000)
$E' + P_i \rightleftharpoons E' \cdot P_i$	K_1 [M^{-1}]	105
$MgE' + P_i \rightleftharpoons MgE' \cdot P_i$	K_3 [M^{-1}]	524
$E + P_i \rightleftharpoons E \cdot P_i$	K_9 [M^{-1}]	100
$Mg_3E + P_i \rightleftharpoons Mg_3E \cdot P_i$	K_{12} [M^{-1}]	450
$E + ATP \rightleftharpoons E \cdot ATP$	– [M^{-1}][b]	$\sim 10^6$
$E' + Mg^{2+} \rightleftharpoons MgE'$	K_2 [M^{-1}]	128
$E' \cdot P_i + Mg^{2+} \rightleftharpoons MgE' \cdot P_i$	K_4 [M^{-1}]	637
$E + Mg^{2+} \rightleftharpoons MgE$	K_6 [M^{-1}]	(1000)
$MgE + Mg^{2+} \rightleftharpoons Mg_2E$	K_7 [M^{-1}]	(200)
$Mg_2E + Mg^{2+} \rightleftharpoons Mg_3E$	K_8 [M^{-1}]	(50)
$E + Ca^{2+} \rightleftharpoons CaE$	– [M^{-1}][c]	$\sim 10^8$
$MgE' \cdot P_i \rightleftharpoons MgE' - P$	K_5	0.93

[a] K values corresponding to scheme of Fig. 2 refer to 25 °C, pH 6.2, no added KCl. Values in parentheses represent values for which only an order of magnitude estimate can be made.
[b] Data of Meissner [17]. This constant is included in the Table to show that the binding of P_i to the E form of the protein would not be able to compete with ATP binding under physiological conditions.
[c] High affinity binding constant for Ca^2 at pH 7, in the presence of 80 mM KCl, based on the data of Inesi *et al.* [5], and the explanation for cooperativity used in the present paper.

lated enzyme at very high [Mg^{2+}], under conditions where no more than a single Mg^{2+} ion can be bound to E′−P. The results cannot (as before) distinguish the conformational state of the enzyme to which these ions are bound. It has however been established that the E form has 3 divalent metal binding sites [9, 10]. One of them (here called β site) is the site that functions as a Mg^{2+} site under normal physiological conditions, but the other two sites (α sites) are the high affinity Ca^{2+} sites, which have only low affinity for Mg^{2+} and can be expected to bind Mg^{2+} only when the Ca^{2+} concentration in the medium has been virtually completely suppressed, as is true for the experiments here reported. Identification of the 3 required Mg^{2+} sites as belonging to the conformational state E is therefore indicated.

The most interesting aspect of these data is the steepness of the inhibition curve, which indicates that Mg^{2+} binding is *operationally* a highly cooperative reaction, and that the species Mg_2E or $Mg_2E \cdot P_i$ could not be a significant fraction of the equilibrium mixture at any concentration of Mg^{2+}. This is the classic hallmark [11] for a binding equilibrium in which a conformational change has to occur before binding (in this case binding beyond the level of one bound Mg per protein molecule in the species $MgE' \cdot P_i$ and MgE′−P). In terms of the scheme of Fig. 2 this means that the equilibrium constant $K_0 = [E']/[E]$ must $\gg 1$. A precise numerical value cannot be obtained from the data without independent *direct* knowledge of Mg^{2+} binding constants. Kalbitzer *et al.* [9] have reported such data, but assumptions that were required to obtain them suggest that they may be subject to considerable error. These data indicate that the affinity of Mg^{2+} for the β site is at least 10-fold higher than the affinity for the two α sites. If we accept this ratio (but not the absolute values of the binding constants), statistical treatment of our results yields $K_0 = 10^3$ as the most probable value and $K_0 = 50$ as a lower limit. If we use the actual numerical values of Kalbitzer *et al.*, and assume that they refer to the E state of the protein, we obtain $K_0 = 3 \times 10^4$. No matter what is assumed about the Mg^{2+} sites, there is an absolute lower limit of $K_0 = 11$.

The course of inhibition by Mg^{2+} was determined at several concentrations of P_i, and this factor was found to have little influence on the results, which requires that P_i must be able to bind to the E state of the protein as well as to the E′ state, with a binding constant of similar magnitude. This is the reason for inclusion of the species $E \cdot P_i$, $MgE \cdot P_i$, etc. in the scheme of Fig. 2: the results could not be fitted by a reaction scheme that did not include them. Presumably this represents binding of P_i to the ATP binding site of E. Competition between P_i and nucleoside phosphates for the same sites has been reported before [12, 13].

Table II. Enthalpies for binding to E′.

	ΔH [kcal/mol]	
	Epstein *et al.* [7]	Martin and Tanford [3]
$E' + P_i \rightleftharpoons E' \cdot P_i$	−23	+12
$MgE' + P_i \rightleftharpoons MgE' \cdot P_i$	− 9	− 4
$E' + Mg^{2+} \rightleftharpoons MgE'$	−76	+ 3
$E' \cdot P_i + Mg^{2+} \rightleftharpoons MgE' \cdot P_i$	−35	−13
$MgE \cdot P_i \rightleftharpoons MgE - P$	–	+ 7

Cooperativity in High Affinity Binding of Calcium

A careful study of the binding of Ca^{2+} to the specific high affinity sites of the Ca^{2+} pump protein has been carried out by Inesi *et al.* [5]. Vesicular prep-

Table III. Relation between K_0 and the cooperativity of binding of Ca^{2+} to the unliganded protein.

K_0	$K_{2,\,app}/K_{1,\,app}$	Hill plot slope
10^{-2}	0.25	1.00
1	0.50	1.17
10^2	25	1.82
10^3	250	1.94
10^4	2500	1.98
Inesi *et al.* [5]	420 ± 150	1.9 ± 0.1

arations were used, at pH 6.8, in the absence of P_i or ATP, but in the presence of 80 mM KCl. The binding proved to be highly cooperative, *i.e.*, using the terminology in the original paper, it was found that the binding of the first Ca^{2+} ion induces a conformational change that makes the affinity for the second Ca^{2+} ion much higher. Inesi *et al.* [5] explained this in teems of a complex reaction scheme that involved four distinct conformational states of the pump protein, but the results can equally well be explained without invoking conformational states other than E and E′. If the unliganded protein is predominantly in the state E′ ($K_0 \gg 1$), no high affinity Ca^{2+} binding sites are present. To bind the first Ca^{2+} ion, one must first convert E′ to E, and that makes the experimentally observed value for the first binding constant ($K_{1,\,obs}$) relatively small. For binding of the second Ca^{2+} ion, the protein is already in the right state, and $K_{2,\,obs}$ is therefore much larger than $K_{1,\,obs}$. The ratio of the two observed binding constant, or the slope of a Hill plot, which is an equivalent measure of cooperativity, clearly depends on the value of K_0. One can estimate K_0 from the observed results by using the relations developed for allosteric enzymes by Monod *et al.* [11], and this is done in Table III. The results show that the data of Inesi *et al.* [5], when interpreted in this way, correspond to $K_0 \simeq 10^3$, in good agreement with the value calculated above on the basis of cooperativity of the binding of Mg^{2+} to the same sites.

It should be noted that the calculation in Table III assumes that the two binding sites on E have identical *intrinsic* affinity for Ca^{2+}. If one had assumed that one of the sites had a higher intrinsic affinity than the other, an even larger value of K_0 would have been obtained, because heterogeneity of binding sites by itself would lead to what is often called *negative* cooperativity, *i.e.*, $K_{1,\,obs} > K_{2,\,obs}$ [14].

Discussion

The most important outcome of this work is the demonstration that the equilibrium constant for the interconversion of the two major conformational states of the calcium pump protein favors the state E′, *i.e.*, $[E']/[E] = K_0 \simeq 10^3$. The numerical value is subject to an uncertainty of about one order of magnitude, which does not reflect experimental uncertainty in the data, but is a consequence of the fact that K_0 was estimated indirectly on the basis of the thermodynamics of association of Ca^{2+} or Mg^{2+} with the pump protein under conditions where direct distinction between E and E′ could not be made. To obtain a more precise value would require measurements that can distinguish directly between the two states. An unambiguous method does not as yet exist.

The high Mg^{2+} concentrations used to obtain these data represent unusual conditions. Since the experiments do not directly demonstrate that the conformational state formed under these conditions is actually the E state, it could be argued that some other state of the protein is involved, one that is not normally encountered in experiments done under conditions closer to the physiological norm. If this were so, however, this new state would have to be more stable than E, and the true equilibrium ratio [E′]/[E] would have to be even larger than the K_0 values reported above. The similarity between the K_0 values obtained on the basis of the Ca^{2+} binding and Mg^{2+} inhibition data also argues against involvement of a different conformation, since E is by definition the conformational state that is favored by Ca^{2+} binding.

The conditions used in our experiments (pH 6.2, absence of KCl) were chosen to maximize the yield of MgE′−P, but the Ca^{2+} binding data of Inesi *et al.* (pH 7.0, 80 mM KCl) more nearly represent physiological conditions. It is reasonable to assume that $[E']/[E] \sim 10^3$, to within an order of magnitude, applies to the physiological state of the pump. The corresponding free energy difference between the E and E′ states of the protein is only 4 kcal/mol, and this is much less than the 14 kcal/mol that is available from ATP hydrolysis [15], and which has to be utilized in some way during each reaction cycle. These figures place obvious limitations on the kind of free energy transduction mechanism one can visualize for the overall active transport process. Storage of free energy by the protein itself, in the manner suggested by

the "rack" mechanism of Lumry [16], can clearly not play a major role.

The ΔH values of Table II are also of interest in relation to the free energy transduction mechanism, because they appear to eliminate the suggestion of Racker [8] that the binding energy of Mg^{2+} plays a major role.

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