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Thermodynamic and kinetic cooperativity in ligand binding to multiple sites on a protein: Ca^{2+} activation of an ATP-driven Ca pump

(active transport/enzyme kinetics/Ca ATPase)

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ABSTRACT **Contrary to common belief, theoretical analysis does not predict any necessary relationship between cooperativity in the equilibrium binding of an ion to multiple binding sites on a protein and cooperativity in the kinetic activation of a reaction for which such binding is prerequisite. The sarcoplasmic reticulum Ca pump protein, for example, has two high-affinity binding sites for Ca^{2+}, here considered to be nearly identical and independent. Equilibrium binding to these sites can be highly cooperative in spite of site-independence, as demonstrated by the well-known allosteric mechanism based on Wyman's principle of linked functions. We show in this paper that kinetic activation of the pump reaction cycle by binding of Ca^{2+} to these same sites can likewise be a cooperative function of Ca^{2+} concentration but that the criteria that determine cooperativity in the two situations are different. It is possible to observe kinetic cooperativity without concomitant cooperativity in equilibrium binding and vice versa. Application of these theoretical considerations to experimental data for the pump protein raises questions about the Ca^{2+} binding mechanism.**

The sarcoplasmic reticulum Ca pump protein has two high-affinity binding sites for Ca^{2+}, treated frequently as indistinguishable in their binding characteristics and by some authors as inherently independent of each other (1, 2). Equilibrium binding to these sites is nevertheless a highly cooperative function of the free Ca^{2+} concentration (3, 4). This result is not incompatible with a lack of direct interaction between the sites. The pump protein has two principal conformational states (E_1 and E_2) and the Ca^{2+} binding sites have high affinity for Ca^{2+} only in the E_1 state, binding affinity being several orders of magnitude smaller in the E_2 state (5). Under these conditions, the conventional allosteric mechanism for generation of cooperativity can come into play, as illustrated by the cooperative association of oxygen with the four virtually identical and inherently independent binding sites of hemoglobin (6, 7).

Binding of Ca^{2+} to the same two sites in the E_1 state of the pump protein is essential for activation of ATP hydrolysis. Although there is some diversity in the available experimental data (see *Discussion*), the hydrolysis rate is usually also reported to be a highly cooperative function of Ca^{2+} concentration (1, 2), and this result is sometimes accompanied by the assertion that such similarity between equilibrium and kinetic manifestations of cooperativity is theoretically expected. This paper addresses itself to this latter assertion. We report a theoretical analysis of the problem, which demonstrates that different parameters determine cooperativity in the two situations, so that no general correspondence between equilibrium and kinetic cooperativities can exist.

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Kinetic equations for any protein-catalyzed reaction are directly related to the catalytic reaction cycle, and it is therefore necessary to assume an explicit model for the Ca pump reaction cycle if kinetic cooperativity is to be quantitatively discussed. We here assume a mechanism for the Ca pump cycle that was suggested in the first paragraph of this paper, namely one involving equivalence and independence of the two Ca^{2+} binding sites in the E_1 state of the protein and allosteric generation of cooperativity in equilibrium binding. Our conclusions therefore apply directly only to this simple model, but this is sufficient to demonstrate the lack of a general relationship between the two kinds of cooperativity.

Model for the Pump Cycle

For detailed analysis, we use the reaction cycle for the Ca pump shown in Fig. 1, essentially the same as the cycle originally introduced by de Meis and Vianna (5). The transport protein alternates in the course of the cycle between conformational states E_1 and E_2. E_1 has two Ca^{2+} binding sites (of high affinity, as already noted), which *in vivo* face the cytoplasmic side of the membrane; it also has a nucleotide binding site with high affinity for ATP. E_2 has two Ca^{2+} binding sites (with low binding affinity), which *in vivo* face the sarcoplasmic reticulum lumen; it also has a binding site for inorganic phosphate, to which ATP presumably can bind with low affinity, as discussed in a previous paper (8). The cycle as here drawn allows the binding of Ca^{2+} and ATP to E_1 to occur in random order, a feature implicit in the de Meis mod-

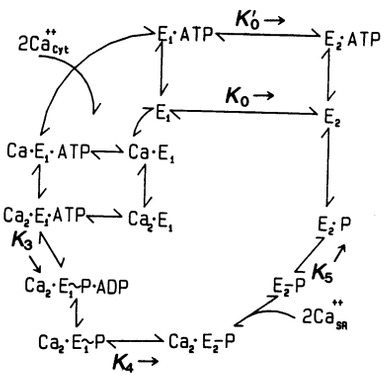

FIG. 1. Conventional reaction cycle for the sarcoplasmic reticulum Ca pump. E_1 and E_2 are distinct conformational states, as described in the text. The arrows for the cycle transitions that do not involve ligand binding indicate the directions of reaction for the corresponding equilibrium constants of Table 1, and for the rate constants k_3, k_4, and k_5. The cycle as here presented does not explicitly incorporate essential roles played by Mg^{2+} and K^+. The calculations are intended to apply to experimental conditions where these ions are present at concentrations that satisfy the requirements. Ca^{++}_{Cyt} and Ca^{++}_{SR} represent Ca^{2+} on the cytoplasmic and lumen sides of the sarcoplasmic reticulum membrane, respectively.

el but not explicitly indicated by him. An important feature of the model in relation to cooperativity is that both Ca^{2+} sites and the ATP site of E_1 must be saturated before formation of the phosphoenzyme intermediate $Ca_2E_1{\sim}P$ can take place.

As previously indicated, the two high-affinity Ca^{2+} binding sites in E_1 are treated as identical and noninteracting. The same statement applies to the low-affinity sites in E_2, but the E_2 sites in fact are not involved in our analysis, which is confined to Ca^{2+} concentrations too low for significant occupancy of these sites.

Equilibrium Binding

Equilibrium binding studies are normally carried out in the absence of ATP or P_i, and thus involve only four of the states of Fig. 1, namely E_2, E_1, CaE_1 and Ca_2E_1. With the assumption of two identical binding sites, only two parameters determine the extent of binding at low Ca^{2+} concentrations: K_{Ca}, the intrinsic binding constant for Ca^{2+} to either site on E_1, and $K_0 = [E_2]/[E_1]$, the equilibrium constant for interconversion of the two conformational states. The degree of saturation of the binding sites (Θ) is given by

$$\Theta = \frac{K_{Ca}C(1 + K_{Ca}C)}{K_0 + (1 + K_{Ca}C)^2}, \quad [1]$$

where C is the free Ca^{2+} concentration in equilibrium with the protein. The Hill coefficient (n_H) at the midpoint of the binding isotherm serves as the most convenient measure of cooperativity. By definition,

$$n_H = d\{\ln[\Theta/(1 - \Theta)]\}/d(\ln C), \quad [2]$$

which, combined with Eq. 1 gives

$$n_H = 1 + \frac{K_0K_{Ca}C}{(1 + K_{Ca}C)(1 + K_{Ca}C + K_0)}. \quad [3]$$

At the midpoint of the binding curve ($\Theta = 0.5$), Eq. 1 gives $(K_{Ca}C)^2 = 1 + K_0$, from which it follows that n_H at the midpoint is given by

$$n_H = 1 + K_0/[1 + (1 + K_0)^{\frac{1}{2}}]^2; \quad [4]$$

i.e., n_H is determined entirely by the value of K_0. We have $n_H = 1.82$ when $K_0 = 100$, $n_H = 1.94$ when $K_0 = 1000$, etc.

It is important to the specific illustrative reaction we are using (but not to the main theme of the paper) that kinetic data for activation of the pump cycle are necessarily obtained in the presence of ATP. Usually a high concentration, close to saturation of ATP binding sites, is used when the effect of changing $[Ca^{2+}]$ is to be measured. Under these conditions, the model of Fig. 1 predicts a decrease in n_H for equilibrium binding of Ca^{2+} to the Ca pump protein, because ATP binds more tightly to E_1 than to E_2 and thereby shifts the equilibrium between the two states (6). In the presence of sufficient ATP to saturate the binding sites of both conformational states, the species present at equilibrium would be $E_2{\cdot}ATP$, $E_1{\cdot}ATP$, $CaE_1{\cdot}ATP$, and $Ca_2E_1{\cdot}ATP$. Eqs. 1–4 would apply as before, with K'_{Ca} (binding constant for Ca^{2+} binding to $E_1{\cdot}ATP$) in place of K_{Ca} and $K'_0 = [E_2{\cdot}ATP]/[E_1{\cdot}ATP]$ in place of K_0. The equilibrium constants for ATP binding to E_1 and E_2 probably differ by a factor of about 1000 (8), which would make $K'_0 = K_0/1000$. Thus, $K'_0 = 0.1$ (leading to $n_H = 1.02$) when $K_0 = 100$, etc.

Equilibrium binding measurements that might test this predicted alteration in n_H have not been reported. Pure binding measurements in the actual presence of ATP would require prevention of initiation of the hydrolysis cycle via $Ca_2E_1{\sim}P$, which is not feasible in the presence of Mg^{2+} levels at which kinetic data are normally obtained. However, tight-binding analogues of ATP that cannot be hydrolyzed could presumably be used.

Kinetic Cooperativity

We derive explicit kinetic equations only for a simple limiting case: ATP hydrolysis at saturating levels of ATP, in the absence of ADP or P_i, with $[Ca^{2+}] = 0$ on the discharge side of the membrane. We further assume that ATP binding and ADP and P_i dissociation are fast compared to the overall rate. These assumptions lead to a simple equation for the steady rate of hydrolysis corresponding to the reaction cycle of Fig. 1,

$$V = \frac{C^2}{\alpha + \beta C + \gamma C^2}, \quad [5]$$

where C is the concentration of Ca^{2+} on the uptake side of the membrane, as before, and

$$\alpha = \frac{1 + K'_0}{K'_{Ca}}\frac{1}{2k_{on}} + \frac{1}{k_3K'_{Ca}}, \quad [6]$$

$$\beta = \frac{3 + K'_0}{2k_{on}} + \frac{2}{k_3K'_{Ca}}, \quad [7]$$

and

$$\gamma = 1/V_{max}. \quad [8]$$

In these relations, K'_0 and K'_{Ca} have the same meaning as before, k_3 is defined by Fig. 1, and k_{on} is the second-order intrinsic rate constant for association between Ca^{2+} and its binding site on E_1. (The rate constant for formation of $CaE_1{\cdot}ATP$ from $E_1{\cdot}ATP$ would have twice this value because two sites are available for binding.)

The parameter γ involves other rate constants of the reaction cycle, but their explicit relation to γ is not needed here because $1/\gamma$ is the maximal rate attainable under the given conditions at high C, and this maximal velocity (V_{max}) is a sufficient parameter in the context of analysis by Eq. 5.

The kinetic Hill coefficient, in analogy to the thermodynamic coefficient, is defined by Eq. 2, but Θ here represents the fractional "saturation" of the steady-state hydrolysis rate, i.e.,

$$\Theta = V/V_{max}, \quad [9]$$

instead of fractional saturation of binding sites. It follows from Eq. 5 that

$$n_H = 1 + \frac{\alpha}{\alpha + \beta C}. \quad [10]$$

At the midpoint,

$$C = C_{\frac{1}{2}} = \frac{\beta}{2\gamma}\left[1 + \left(1 + \frac{4\alpha\gamma}{\beta^2}\right)^{\frac{1}{2}}\right] \quad [11]$$

and the midpoint Hill slope becomes

$$n_H = 1 + \frac{F}{(1 + F) + (1 + 2F)^{\frac{1}{2}}}, \quad [12]$$

where

$$F = 2\alpha\gamma/\beta^2. \quad [13]$$

These relations yield only meager kinetic cooperativity when $F < 1$. At higher values of F, we have $n_H = 1.27$ for $F = 1$, $n_H = 1.64$ for $F = 10$, and $n_H = 1.87$ for $F = 100$.

To obtain numerical values for F and the resulting n_H, we need to specify the parameters that determine α and β. For purpose of illustration, suppose that $k_3K'_{Ca} >> k_{on}$, which is actually a reasonable approximation for the Ca pump. This inequality allows us to neglect the last terms in Eqs. 6 and 7, giving

$$F = \frac{(1 + K'_0)k_{on}}{(3 + K'_0)^2 V_{max} K'_{Ca}}. \quad [14]$$

Eq. 14 shows that under these illustrative conditions, a shift in the conformational equilibrium towards state E_2 (i.e., an increase in K'_0) does not promote kinetic cooperativity, in contrast to the result for equilibrium binding cooperativity given by Eq. 4. On the other hand, the equation shows that kinetic cooperativity is possible even when equilibrium binding is not cooperative. For example, we noted previously that the model used here predicts that equilibrium binding in the presence of ATP should not be significantly cooperative, with K'_0 likely to be well below unity. With a low value for K'_0 and $K'_{Ca} = 10^6\ M^{-1}$, $V_{max} = 3\ sec^{-1}$, and $k_{on} = 3 \times 10^8\ M^{-1}\ sec^{-1}$ (all quite reasonable values), Eq. 14 gives $F = 12.5$, which, by Eq. 5, leads to $n_H = 1.67$.

Fig. 2 demonstrates the same result in graphical form and without the simplifying assumptions used for the formal equations above. The curves shown were obtained from numerical solutions of the rate equations for the complete cycle of Fig. 1, under conditions that simulate experimental studies of the activation of ATP hydrolysis in leaky sarcoplasmic reticulum vesicles. A constant value of $K_0 = 100$ was used for these calculations, yielding $n_H = 1.82$ for equilibrium binding in the absence of ATP. The equilibrium constant for binding of ATP to E_1 was set at least 1000 times larger than the equilibrium constant for binding to E_2, which makes $K'_0 < 0.1$ for all the calculations and $n_H \leq 1.02$ for equilibrium binding in the presence of ATP, independent of other parameters. The calculated curves show that the *kinetic* Hill coefficient can assume almost any value between 1 and 2, depending on values assigned to the other parameters of the cycle (Table 1). The value of k_3 was set relatively high for all three of the curves, so that the condition leading to Eq. 14 is approximately satisfied. As expected from this equation, k_{on} and K'_{Ca} then become particularly important in the determination of n_H.

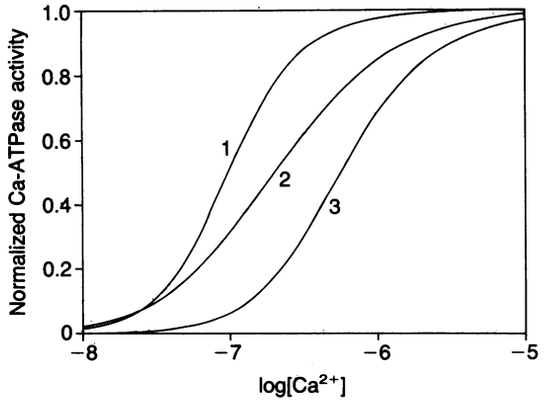

FIG. 2. ATP hydrolysis in leaky vesicles as a function of free Ca^{2+} concentration. The curves are theoretical, based on the complete reaction sequence of Fig. 1, with minor extensions to allow for random order of dissociation of products on the right-hand side. The rate and equilibrium constants used for the calculations are given in Table 1. Maximal turnover rates are 3.4, 5.8, and 8.6 sec^{-1}, respectively, for the three curves. Kinetic Hill coefficients at the activation midpoints are 1.82, 1.11, and 1.43, respectively.

In addition to the equilibrium and rate constants used for the present calculations, Table 1 also lists the corresponding parameter values that were used by us previously (8) for calculation of the substrate activation of ATP hydrolysis at constant $[Ca^{2+}]$.

It should be noted that all three curves in Fig. 2 lead to a decrease in hydrolysis rate (not shown) when extended to higher $[Ca^{2+}]$, a result due to the fact that the curves were calculated for leaky-vesicle experiments in which $[Ca^{2+}]$ is the same on both sides of the membrane and thus able to inhibit Ca^{2+} release by binding to the low-affinity binding sites in E_2-*P*. A similar decrease is observed in experimental studies when leaky vesicles are used. Most of the differences that we have employed for the central group of "other equilibrium constants" shown in Table 1 arise from the need to keep the binding constant for Ca^{2+} to these low-affinity sites at a reasonable level to preserve this inhibitory effect, while at the same time satisfying the general thermodynamic requirement that the product of all equilibrium constants around the cycle must remain constant (equal to the equilibrium constant for hydrolysis of ATP) when K'_{Ca} is altered.

Extension of our analysis to situations not directly dealt with in this paper indicates that a lack of interdependence of equilibrium and kinetic cooperativities is the normally expected result. For the scheme of Fig. 1, for example, with no restrictions on rate and equilibrium constants, correspondence between equilibrium and kinetic cooperativities is theoretically expected only when k_3 is the rate-limiting step of the overall reaction (i.e., $k_3 \approx V_{max}$). In that event, the immediate precursors of the slow step would be close to equilibrium with each other, and the overall rate would be determined by the product of k_3 and the equilibrium fraction of total E_1 that exists in the state $Ca_2E_1 \cdot ATP$.

Levels of Cycle Intermediates

As is to be expected, there is a correlation between the levels of cycle intermediates and the calculated degree of cooperativity; i.e., species with a single bound Ca^{2+} become significant only at low degrees of cooperativity. The maximal fraction of total protein in the state ($CaE_1 + CaE_1 \cdot ATP$) at any stage of the activation process is 0.40 for curve 2 ($n_H = 1.11$), 0.23 for curve 3 ($n_H = 1.43$), and 0.075 for curve 1 ($n_H = 1.82$). This establishes a parallel between the criteria for equilibrium and kinetic cooperativities. The difference, of course, is that we are dealing with intermediate levels at equilibrium in one case and at steady state during rapid cycling in the other.

DISCUSSION

The primary objective of this paper is to demonstrate the lack of any necessary relationship between cooperativity in the equilibrium binding of Ca^{2+} to the sarcoplasmic reticulum Ca pump protein and kinetic cooperativity in the Ca^{2+} activation of ATP hydrolysis. The result obtained should in principle be quite general, applicable to any enzyme or transport protein that requires more than one molecule of a ligand for kinetic activation.

We used the sarcoplasmic reticulum Ca pump protein as an example for the analysis, which required adoption of a reaction sequence (Fig. 1) for the overall pump reaction. It should be made clear that this particular reaction scheme is not definitely established. In particular, it is worth noting that there exist transient kinetic data (9) that have been interpreted as suggesting that one Ca^{2+} ion can bind to the pump protein from the cytoplasmic side of the membrane before

Table 1. Equilibrium and rate constants for calculated curves (see Fig. 2)

	Curve 1 (n_H = 1.82)	Curve 2 (n_H = 1.11)	Curve 3 (n_H = 1.43)	Ref. 8* (n_H = 1.26)
Parameters important for determining cooperativity				
K'_0	0.025	0.1	0.025	0.08
K_0	100	100	100	100
K'_{Ca} (M^{-1})	1×10^6	2×10^7	1×10^6	1×10^7
V_{max} (sec^{-1})	3.4	5.8	8.6	7.8
k_{on} ($M^{-1}sec^{-1}$)	3×10^8	5×10^7	4×10^7	1.5×10^8
k_3 (sec^{-1})	2000	1000	1000	1000
Other equilibrium constants				
K_b, Ca^{2+} to E_2–P	200	400	200	200
K_b, ATP to E_1	4×10^6	1×10^6	4×10^6	1×10^6
K_b, ATP to E_2	1000	1000	1000	800
K_b, ADP to E_1~P	1000	5000	1000	5000
K_b, P_i to E_2	400	600	400	600
K_3	0.4	0.4	0.4	0.4
K_4	0.24	0.09	0.24	0.09
K_5	1	0.8	1	0.8
Other rate constants				
E_2·ATP → E_1·ATP (sec^{-1})	6	10	15	17
E_2 → E_1 (sec^{-1})	1.2	2	3	2.5
k_4 (sec^{-1})	10	20	30	38
k_5 (sec^{-1})	500	400	500	400
k_{on} (ATP) ($M^{-1}sec^{-1}$)	1×10^7	1×10^7	1×10^7	1×10^6

*Parameters used to describe ATP activation at constant [Ca^{2+}].

transition to the E_1 state, a possibility that is in no way compatible with Fig. 1.

Existence of this kind of reservation about the basic mechanism makes it instructive to look for possible inconsistencies between calculated results and experimental data for the Ca pump protein. Experimental steady-state kinetic data from different laboratories unfortunately differ quite widely (1, 2, 10–16). For example, n_H values as low as 1.0 have been reported, as well as values "close to 2." Similarly, midpoints of reported activation curves vary from 8×10^{-8} to 1×10^{-6} M under similar experimental conditions. As Fig. 2 shows, it is possible to obtain theoretical curves from the model of Fig. 1 that can reproduce results within these ranges, and the kinetic and thermodynamic parameters that must be used to do so (Table 1) are inherently reasonable. There is, however, one parameter of concern when one wishes to account for n_H values in the upper part of the reported range ($n_H > 1.5$), and this is the equilibrium constant for binding of Ca^{2+} to its high-affinity sites on E_1. If cooperativity of equilibrium binding is attributed to the linked-function principle, as we have done in using the model of Fig. 1, then the minimal value for K_{Ca} from binding studies in the absence of nucleotides at room temperature is 10^7 M^{-1} (17). It has been suggested in the literature that there is no direct effect of nucleotides on Ca^{2+} binding (15, 18), which would mean that K'_{Ca} should also be assigned a value $\geq 10^7$ M^{-1}. However, the values of K'_{Ca} that are needed to obtain kinetically cooperative activation plots (as in curve 1 of Fig. 2) are close to 10^6 M^{-1}. With the higher value of 10^7 M^{-1} for this binding constant, theoretical Hill slopes are limited to about 1.3 or less, as in curve 2 of Fig. 2.

It is worth noting in this connection that the parameters in the last column of Table 1, which very closely reproduce the ATP-activation data of Møller *et al.* (15), also correctly predict the midpoint for Ca^{2+} activation reported by the same investigators. The calculated midpoint Hill slope, however, is 1.26, compared to the reported experimental value of 1.75.

These results can be understood in terms of Eq. **14**, which, though derived on the basis of simplifying assumptions, can approximately predict the numerically calculated results of Fig. 1. Eq. **14** allows compensation for a change in K'_{Ca} only by alteration in k_{on} or V_{max}. The calculation in the text that led (with $K'_{Ca} = 10^6$ M^{-1}) to $F = 12.5$ and $n_H = 1.67$ would instead lead to $F = 1.25$ and $n_H = 1.3$ if we set $K'_{Ca} = 10^7$ M^{-1} without other change. We cannot make a sufficiently large compensating increase in k_{on}, because the original value (3×10^8 M^{-1} sec^{-1}) is already close to the upper limit for diffusion-controlled binding (19). Likewise, the original value of V_{max} that was used (3 sec^{-1}) is smaller than most literature reports by a factor of 2 to 3 and therefore needs to be increased rather than decreased, which results in a further decrease in the calculated n_H.

The lack of self-consistency between experimental data for Ca^{2+} activation of the pump reaction may result from different methods of calculation of free Ca^{2+} concentration in Ca/EGTA buffer systems, as has been suggested before (2), or from the use of such buffer solutions with insufficient buffering capacity throughout the Ca^{2+} concentration range for which they were employed. Whatever the cause, a meaningful test of theoretical results based on the mechanism of Fig. 1 for the sarcoplasmic reticulum Ca pump must await consensus on the experimental data. Our theoretical demonstration that equilibrium binding cooperativity and kinetic cooperativity do not have a necessary relationship is, of course, not limited to this particular system or the mechanism assigned to it and, therefore, does not depend on attainment of such consensus.

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