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Application of the principle of linked functions to ATP-driven ion pumps: Kinetics of activation by ATP

(active transport/enzyme kinetics/ligand binding/Na^+,K^+-ATPase/Ca^{2+}-ATPase)

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ABSTRACT **If a ligand binds with unequal affinity to two distinct states of a protein, then the equilibrium between the two states becomes a function of the concentration of the ligand. A necessary consequence is that the ligand must also affect the forward and/or reverse rate constants for transition between the two states. For an enzyme or transport protein with such a transition as a slow step in the catalytic cycle, the overall rate also becomes a function of ligand concentration. These conclusions are independent of whether or not the ligand is a direct participant in the reaction. If it is a direct participant, then the kinetic effect arising from the principle of linked functions is distinct from the direct catalytic effect. These principles suffice to account for the biphasic response of the hydrolytic activity of ATP-driven ion pumps to the concentration of ATP, without the need to invoke more than one ATP binding site per catalytic center.**

Wyman's "Principle of Linked Functions" (1) follows rigorously from basic thermodynamic conservation laws. One of its consequences is that the equilibrium between two conformational states of a protein must be a function of the concentrations of any ligands that can bind to the two states with unequal binding affinities. Being a thermodynamic principle, it has in the past normally been applied to purely equilibrium situations, the classic examples being the Bohr effect, the effect of pH on the equilibrium between oxygen and hemoglobin (1), and "allosteric" enzymes, where the principle is applied to the equilibrium between two states of an enzyme that differ in their reactivities (2).

It is, however, self-evident that one cannot change the equilibrium constant for a reaction without changing at least one of the two (forward and reverse) corresponding rate constants. In the absence of some compelling reason to the contrary, both forward and reverse rate constants are likely to be affected. Herein lies the novelty in this paper: an examination of the unavoidable kinetic effects of the linked-function principle. The theoretical aspects of the paper are quite general but will be presented with reference to a particular system, ATP-driven Ca and Na/K pumps.

Reaction Cycles of Ion Pumps

The catalysis of active transport requires cycling of the transport protein between a minimum of two distinct conformational states, in which binding sites for the transported species face opposite sides of the membrane (3–7). In the case of ATP-driven Ca and Na/K pumps the two conformations are generally called E_1 (or E) and E_2 (or E' or E^*), respectively, E_1 being the "uptake" conformation in which the binding sites for the ion to be extruded face the cytoplasmic side of the membrane. The transitions between these conformations occur twice in each reaction cycle, in one direction when the protein is phosphorylated and in the other direction when it is not phosphorylated. Both transitions have been found to be relatively slow steps in the reaction cycle—i.e., they play an important role in control of the overall rate of the transport reaction. Additional conformational states of the protein (other than E_1 and E_2) are often postulated but are not necessary for the analysis of this paper.

The two principal conformations of these proteins differ not only in the orientation of ion binding sites but also in their thermodynamic binding constants for the transported ions and for ATP and its hydrolysis products. In the case of ATP-driven Ca and Na/K pumps, E_1 has a high binding affinity for ATP and for the extruded ions (Ca^{2+} or Na^+), whereas E_2 has a much lower affinity for these ligands (3, 8). Binding of ATP to E_2 is not an essential element of the reaction cycles for these systems. However, E_2 is the discharge state for P_i in ATP hydrolysis and is known to have a site with moderately high binding affinity for P_i from studies of reversal of the pump cycle. It is difficult to imagine a P_i binding site on a protein for which ATP would not compete, so that it is expected that ATP should be able to bind to E_2 with low affinity. Therefore ATP should affect the rate of the $E_1 \rightleftharpoons E_2$ transition (quite apart from its direct effect on the overall rate as a reaction substrate), and this provides a good illustration of the theoretical consequences of the linked-function principle. Promotion of the conversion of E_2 to E_1 by ATP has been known for a long time, but its origins have so far been controversial (3, 8–10).

Fig. 1 shows the derivation of the linkage of equilibrium constants for this system. It is evident that $K_1/K_0 = L_1/L_2$, and, given that E_1 has the high affinity (catalytic) binding site for ATP, we here expect $L_1 > L_2$. It follows that increasing ATP concentration must shift the equilibrium between E_1 and E_2 toward E_1.

To convert this purely thermodynamic relation to a kinetic one, we introduce the four rate constants for the conformational transition between E_1 and E_2, k_{0+} and k_{0-} for unliganded protein ($k_{0+}/k_{0-} = K_0$) and k_{1+} and k_{1-} for ATP-complexed protein ($k_{1+}/k_{1-} = K_1$), a plus sign designating the direction from E_2 to E_1. This leads to

$$\frac{k_{1+}}{k_{0+}} \cdot \frac{k_{0-}}{k_{1-}} = \frac{L_1}{L_2}, \qquad [1]$$

from which, with $L_1 > L_2$, we see that ATP must either accelerate the conversion of E_2 to E_1 ($k_{1+} > k_{0+}$) or diminish the rate of the reverse reaction ($k_{0-} > k_{1-}$) or both.

The simplest way to consider the relative magnitudes of the two rate constant quotients on the left-hand side of Eq. 1 is by use of the Eyring free energy profile (11) along the reaction pathway, shown in Fig. 2. The difference in standard free energy between E_1 and E_1·ATP (δG_1) is larger than the corresponding difference (δG_2) for E_2. The difference δG^* at the peak of the free energy barrier (activated complex) determines the relative rate constants. In the absence of specific

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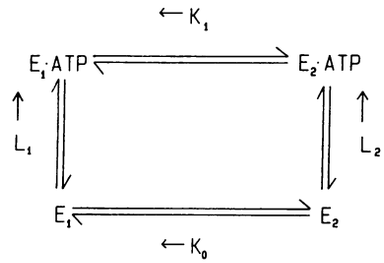

FIG. 1. Equilibrium constants for the $E_1 \rightleftharpoons E_2$ transition. L_1 and L_2 are the binding constants for ATP in the two conformations ($L_1 > L_2$); K_0 is the equilibrium constant for the transition from bare E_2 to bare E_1; K_1 is the equilibrium constant for this transition when ATP is bound. In the case of Na/K pumps, two K^+ ions are transported across the membrane as part of the transition.

indications to the contrary, δG^* is assumed to be intermediate between δG_1 and δG_2,

$$\delta G^* = \alpha\, \delta G_1 + (1 - \alpha)\, \delta G_2, \qquad [2]$$

where α is between 0 and 1. With this relation,

$$\frac{k_{1+}}{k_{0+}} = \left(\frac{L_1}{L_2}\right)^{\alpha} \qquad \frac{k_{0-}}{k_{1-}} = \left(\frac{L_1}{L_2}\right)^{1-\alpha}. \qquad [3]$$

A similar equation occurs frequently in theoretical work dealing with the effect of membrane potential on the kinetics of ion transport (12). In that case, α has usually been set equal to 0.5.

The overall rate coefficients for the transition from E_2 to E_1 (k_f) or the reverse (k_r) depend on the fraction of protein molecules with bound ATP. In the simplest possible case, where ATP binding and dissociation are fast compared to the rate of the conformational transition, ATP binding becomes an equilibrium property, and we have

$$k_f = \frac{L_2[\mathrm{ATP}]\, k_{1+} + k_{0+}}{1 + L_2[\mathrm{ATP}]} \qquad [4]$$

$$k_r = \frac{L_1[\mathrm{ATP}]\, k_{1-} + k_{0-}}{1 + L_1[\mathrm{ATP}]}. \qquad [5]$$

These relations show that the observable kinetic enhancement depends not only on the ratio L_1/L_2 (as in Eq. 3) but also on the individual values of the two binding constants. For example, if L_2 becomes insignificantly small in comparison to L_1, then the ratio k_{1+}/k_{0+} will become extremely large, but enhancement of k_f arising from this effect will not be experimentally observable at reasonable ATP concentrations because L_2[ATP] in Eq. 4 will be too small.

Kinetics of Activation of Coupled Transport

Fig. 3 shows the alternate order of ATP binding built into the conventional reaction cycle for the sarcoplasmic reticulum

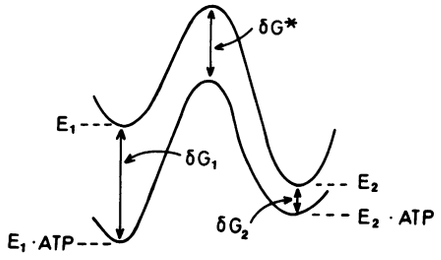

FIG. 2. Free energy profile along the reaction pathway. δG_1 and δG_2 are standard free energies proportional, respectively, to ln L_1 and ln L_2.

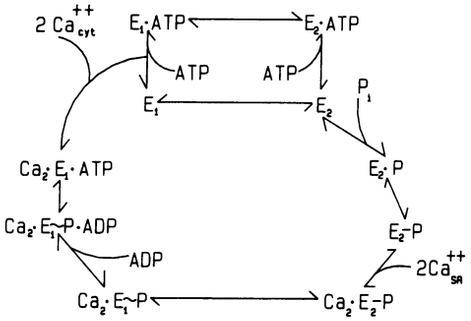

FIG. 3. Skeletal reaction cycle for the sarcoplasmic reticulum Ca pump. $E_1 \sim P$ and E_2-P represent the "high energy" and "low energy" phosphoenzyme intermediates, respectively. The subscripts "cyt" and "SR" refer to Ca^{2+} normally entering or leaving the cycle from the cytoplasmic and sarcoplasmic reticulum sides of the membrane, respectively. Requirements for Mg^{2+} and K^+ are not indicated. Kinetic and thermodynamic parameters for this model are implicitly for conditions where these requirements have been met. Calculations based on this model permitted Ca^{2+} and ATP to bind to E_1 in random order.

Ca pump (3). The well-known Post–Albers scheme (not shown) represents a similar cycle for Na/K pumps (6, 7). For examination of the expected effects of rate constant enhancement, we have limited ourselves to the ATP dependence of ATP hydrolysis (or ion fluxes coupled to it), in the virtual absence of ADP or P_i, where the reverse steps involving ADP and P_i binding (and thereby the reverse component of the overall reaction) can be set equal to zero. The transition $E_2 \rightarrow E_1$ is probably the slowest step under these conditions, except when [ATP] falls to very low levels. In that case ATP binding can become rate limiting, and the assumption of rapid equilibrium for ATP binding (as in the derivation of Eq. 4) has to be abandoned.

In the absence of rate enhancement (e.g., $L_2 = 0$ or $L_1 = L_2$) the scheme of Fig. 3 or the Post–Albers scheme for Na/K pumps would lead to a simple Michaelis–Menten-type equation for the steady-state rate as a function of ATP concentration,

$$V = \frac{a\,[\mathrm{ATP}]}{1 + b\,[\mathrm{ATP}]}, \qquad [6]$$

where a and b are appropriate combinations of rate constants and ion concentrations. Allowance for ATP binding to E_2 and the consequent effect of ATP on the rate of the $E_2 \rightarrow E_1$ transition makes the steady-rate equation more complex, leading to

$$V = \frac{a'\,[\mathrm{ATP}] + b'\,[\mathrm{ATP}]^2}{1 + c'\,[\mathrm{ATP}] + d'\,[\mathrm{ATP}]^2}. \qquad [7]$$

The coefficients a' to d' are readily evaluated by application of the King–Altman (13) procedure to the cycle diagram, or, alternatively, steady-state rate calculations can be made directly by numerical solution of the differential equations for each species.

It is well established that ATP activation data for both the sarcoplasmic reticulum Ca pump and for Na/K pumps from various sources obey Eq. 7 rather than Eq. 6 (9, 14, 15). The question here is then whether this result can be quantitatively accounted for by reasonable values for the parameters needed to produce rate enhancement—i.e., L_2 and α or L_2 and k_{1+}/k_{0+}. [The equilibrium constants L_1 for binding of ATP to E_1 are reasonably well established by existing data (14, 16).]

Comparison with Experiment

The concentration of unbound Mg^{2+} is known to have both stimulatory and inhibitory effects on the rate of ATP hydrolysis, for which there is as yet no agreed upon molecular explanation (17–19). We have chosen to use experimental data for the Na/K pump (Fig. 4) that come from studies in which hydrolysis rate was measured as a function of both ATP and free Mg^{2+} and have plotted the maximal hydrolytic activity obtained at each given total ATP concentration. Under these conditions the balance between stimulatory and inhibitory effects of free Mg^{2+} should be maintained throughout the experimental range of ATP concentrations. In the case of the sarcoplasmic reticulum Ca pump (Fig. 5), where equally extensive data are not available, we arbitrarily selected recent results of Møller *et al.* (14), obtained at a constant concentration of free Mg^{2+} (1 mM). Both figures clearly show the biphasic nature of the substrate dependence of the hydrolysis rate, which necessitates description by Eq. 7.

The theoretical curves in Figs. 4 and 5 are numerical solutions of the rate equations for the cycle of Fig. 3 or the corresponding cycle for the Na/K pump. The parameters for ATP interaction (shown in Table 1) were treated as adjustable. Equilibrium and rate constants for other steps in the cycles were held constant. Actual values were based on experimental data from different sources and will be discussed in detail in subsequent papers.

The data of Møller *et al.* (14) and earlier results of Neet and Green (9) were originally published in the form of double-reciprocal plots. Fig. 6 shows the theoretical curve of Fig. 5 replotted in this way to facilitate direct comparison.

The important aspect of our results is that the parameters in Table 1 required for the theoretical fit are entirely reasonable. The values of L_1 are based on published binding studies (14, 16). The values of L_2 are reasonable for association with a low-affinity site that is likely to be designed for P_i rather than ATP. The association constant between P_i and the E_2 state (with Mg^{2+} concentration in the millimolar range) is about 500 or 600 M^{-1} for the Ca pump (20) and probably has a similar value for Na/K pumps, and it is not unexpected that the binding constants for ATP should be slightly larger than this because of the higher negative charge of the ligand. The values of α between 0.25 and 0.5 show that rate enhancement in the forward direction is relatively modest in comparison with the ratio L_1/L_2 and indicate that the thermodynamic force resulting from the stronger binding of ATP to E_1 is somewhat more effective in resisting the transition from E_1 to E_2 than it is in "pulling" E_2 toward E_1.

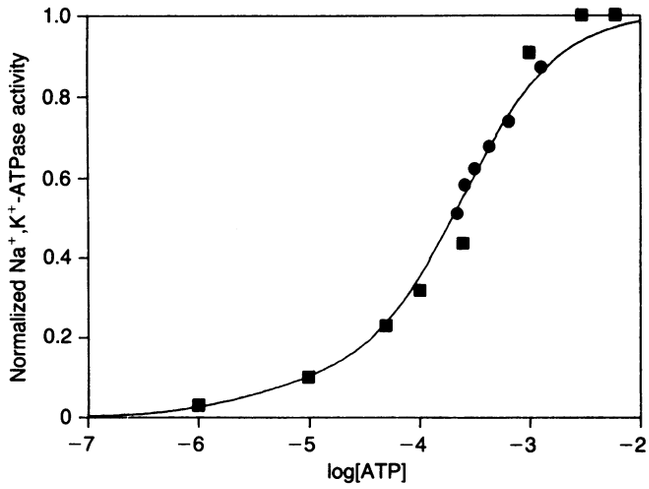

FIG. 4. ATP hydrolysis rate as a function of ATP concentration (molar units) for Na/K pumps at 37°C, pH 7.5. Experimental data from ref. 17 (■) and ref. 18 (●). The curve is theoretical, using the parameters of Table 1. See text for details.

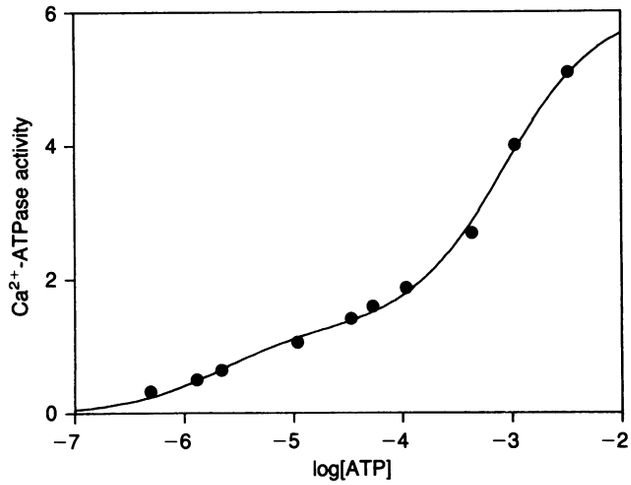

FIG. 5. ATP hydrolysis rate (μmol/mg of protein per min) as a function of ATP concentration (molar units) for the sarcoplasmic reticulum Ca pump at 20°C, pH 7.5. Experimental data from ref. 14, with a theoretical curve based on the parameters of Table 1.

It should be noted that the experimental data encompass very low ATP concentrations at which the velocity of ATP binding steps becomes comparable to the overall rate of the cycle even if the rate of binding is assumed to be diffusion controlled. Under these conditions it is not valid to assume rapid equilibrium for ATP binding in any theoretical model. The rate constants required for good fit (k_{on} in Table 1) are a little smaller than rate constants for diffusion-limited binding of a small symmetrical ligand, a reasonable result considering the likely spatial restrictions on the approach of a complex molecule like ATP to a binding site tailored for high-affinity binding.

Discussion

The kind of kinetic plots seen in Figs. 4–6 suggests the possible existence of two distinct ATP binding sites, a high-affinity site for catalysis of hydrolysis and a site of lower affinity for "activation." That similar plots can be obtained without invoking two sites was first proposed several years ago (explicitly for the sarcoplasmic reticulum Ca pump) by Neet and Green (9), who pointed out that biphasic activation could result from a single binding site if this site has different affinities in two states of the protein linked by a slow transition. More recently, Moczydlowski and Fortes (15) made a similar proposal (explicitly for Na/K pumps) and used a model similar to the model used in this paper for calculation of activation curves. However, neither paper indicated the *thermody-*

Table 1. Parameters for theoretical curves

	Ca pump (Fig. 5)	Na/K pump (Fig. 4)
L_1 (equilibrium constant, ATP + E_1), M^{-1}	1×10^6	2×10^6
L_2 (equilibrium constant, ATP + E_2), M^{-1}	800	1750
k_{on} (rate constant, ATP + E_1), $M^{-1}s^{-1}$	1×10^6	5×10^6
k_{1+} (rate constant, E_2·ATP → E_1·ATP), s^{-1}	17	400
k_{0+} (rate constant, $E_2 \rightarrow E_1$), s^{-1}	2.5	14
α (Eq. 3)	0.27	0.48

Parameters are for 20°C for the Ca pump and for 37°C for the Na/K pump. For the latter pump, two K^+ ions are carried across the membrane as part of the $E_2 \rightarrow E_1$ transition.

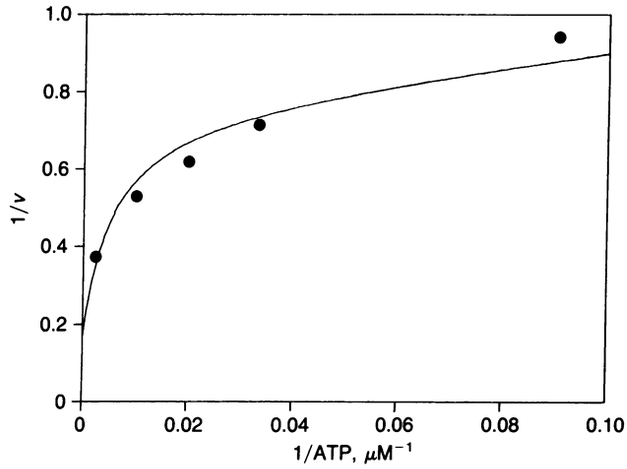

FIG. 6. The theoretical curve of Fig. 5 and in-range experimental points replotted in double-reciprocal form.

namic origin of the observations. Moczydlowski and Fortes in fact violated the basic thermodynamic law (Eq. 1) in their assignment of rate constants to the alternative pathways for ATP binding.

We have demonstrated that biphasic activation based on a single ATP binding site per molecule can in fact be a direct consequence of thermodynamic laws as expressed in the principle of linked functions and is therefore not necessarily a phenomenon that has evolved to fill some regulatory need. This is not intended to imply a lack of functional utility but only to state that the phenomenon would probably be unavoidable for ATP-driven pumps even without functional utility since weak binding of ATP to the P_i release site of state E_2 would seem to be difficult to prevent, and strong binding to E_1 is functionally essential.

We should, in conclusion, make clear that the fixed rate and equilibrium constants (values not given) that we have used as a framework for the calculations of this paper are still subject to change, and even the reaction sequence of Fig. 3 or the Post–Albers scheme should not be considered as definitely established. These uncertainties could, however, affect the parameters of Table 1 only weakly. Our overall conclusions regarding ATP activation are close to being model-independent.

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