APPLICATION OF THE THEORY OF ENZYME SUBUNIT INTERACTIONS TO ATP-HYDROLYZING ENZYMES

The Case of Na,K-ATPase

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ABSTRACT The theory developed by T. L. Hill (1977, *Proc. Natl. Acad. Sci. USA*, 74:3632–3636) for enzyme interactions is applied to a dimeric enzyme, the subunits of which may each exist in three distinct states (as in a uni-bi kinetic mechanism). It is shown that when simultaneous binding of substrate to both subunits is excluded, the complex kinetic mechanism of the dimer reduces to a simpler scheme with two distinct, but analogous, cycles that are in principle separately observable in kinetic experiments. Because of the intersubunit interactions, which are explicitly taken into account, the two cycles have different Michaelis constants and maximal velocities. The model exhibits negative cooperativity and enhanced reactivity, relative to a monomeric enzyme. The theory is applied to Na,K-ATPase for which a complete, bicyclic, kinetic mechanism and rate constants are available. When taken together with other evidence, structural as well as functional, the striking similarity of the observed kinetics with that developed for a dimeric enzyme strongly suggests that the functional unit of Na,K-ATPase is a dimer. The free energy differences (calculated from the known rate constants) between intermediates are 6–16 kJ/mol, comparable, for example, to the free energy associated with the formation of a base pair in a nucleic acid double helix. The possible relevance of these results for other ATPases is briefly discussed.

INTRODUCTION

Na,K-ATPase is the enzyme responsible for the active transport of Na^+ and K^+ across biological membranes. It is a membrane-bound energy transducer, converting free energy derived from the hydrolysis of the substrate ATP into the energy necessary for the vectorial transport of Na^+ and K^+ against their electrochemical gradients.

One of the ways to study this enzyme system is to investigate its ATPase kinetics in fragmented membrane preparations. Obviously, this procedure precludes a study of the ionic transport. Instead, one can observe the influence, on the kinetics of hydrolysis, of the ions Na^+ and K^+, and also of Mg^{++}, whose presence is a necessary prerequisite for function.

When the enzyme protein is purified by delipidation of membrane fragments, the catalytic activity is lost. It can be restored by the addition of lipids. Apparently, the structural properties conferred upon the protein by a lipid matrix is necessary for its function.

From a purely kinetic point of view the enzyme, in any particular ionic environment, exhibits three distinguishable states: the empty enzyme E, the enzyme–substrate adduct ES, and a phosphorylated state EP. Thus the first product to be released in the kinetic mechanism is ADP. Actually several different, but isomeric, phosphoenzymes are known, but this is immaterial for the general form of the rate equation.

There are two distinct regimes for activity, each of which may be satisfactorily described kinetically by a mechanism with the above three enzyme states. They are, respectively, "Na-ATPase conditions," characterized by micromolar substrate concentrations and Na^+, and "Na,K-ATPase conditions," characterized by millimolar substrate concentrations and Na^+ and K^+ present.

In a series of papers (1–3) a detailed analysis of kinetic data has been reported, including the specific influence of Na^+ and K^+ on the kinetics (4, 5). These results lead to the construction of a minimal kinetic model for the enzyme, quantitatively characterized by a complete set of rate constants and equilibrium constants for binding of the inorganic ions to various enzyme intermediates. The main feature of this model (see below) of interest in this connection is that it consists of two connected, but otherwise distinct hydrolysis cycles, each with "its own" K_m and V_{max}. The two cycles contain functionally analogous, but kinetically very different, intermediates. The most widely used kinetic model for Na,K-ATPase is that by Albers (19) and Post et al. (20). It also has two hydrolysis cycles, but they are partially overlapping. Specifically, the step corresponding to release of the first product ADP and the

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resulting phosphoenzyme intermediates are common to both cycles. However, analyses of a large number of steady-state as well as transient kinetic data (2–5, 17, 21–23), pertaining to the membrane-bound enzyme from bovine brain, have demonstrated that the Albers–Post scheme is inadequate as a model for representing all the available kinetic evidence; the Na-ATPase and Na,K-ATPase activities, respectively, have to be represented by two distinct hydrolysis cycles. Briefly, the two main arguments against partially overlapping hydrolysis cycles are: (*a*) For both activities the value of the intrinsic (i.e., cation independent) rate constant characterizing the breakdown (by ADP release) of the enzyme–substrate complex to a phosphoenzyme may be extracted from the experimentally obtained kinetic parameters. The value obtained for Na,K-ATPase is ~30 times larger than that obtained for Na-ATPase (5, 21). Consequently, the product-releasing step for Na-ATPase cannot be the same as that for Na,K-ATPase, nor can it in the latter case be a part of a sequence resulting in product release, because the overall rate through such a sequence cannot exceed that of the slowest step. (*b*) The kinetic competence of the phosphoenzyme first formed in the case of Na-ATPase is insufficient to account for the more than 25-fold increase in activity of Na,K-ATPase, relative to that of Na-ATPAse at 37°C (17, 23).

In view of this situation it seems worthwhile to investigate whether the observed bicyclic kinetic model may be an expression of some physical property of the enzyme unit, thus providing a physical interpretation of analogous, but kinetically very different, enzyme intermediates in the two cycles. This is the purpose of the present work.

One of the still unsettled questions for Na,K-ATPase is whether the functional unit is a monomer or an oligomer. A comprehensive account of the experimental work on this problem has appeared recently (6). Theoretical transport models based on a dimeric enzyme have been published (7, 8), but in none of these has the specific influence of subunit interactions on the kinetics been taken into account.

A formalism for incorporating such effects has been developed by Hill (9) and Hill and Levitzki (10). The present work is an application of this formalism to a dimeric enzyme, the subunits of which can each exist in three distinguishable kinetic states. Thus the results are applicable to any (dimeric) enzyme having a uni-bi mechanism (as for ATPase) or, equivalently, a bi-uni mechanism.

From the results it is clear that the bicyclic scheme derived for the kinetics of Na,K-ATPase (in the presence of both Na^+ and K^+) is precisely the one expected for the action of a dimeric enzyme, with interactions between the subunits that depend on their instantaneous states. The rate constants derived from the kinetic data allow the calculation of the free energy differences between dimer states that are responsible for the enhanced reactivity and decreased substrate affinity observed for the enzyme at larger substrate concentrations and in the presence of K^+. The magnitude of the free energy differences is of the order of the free energy change associated with the formation of a few hydrogen bonds, as, for example, in the formation of a base pair in nucleic acids. On this basis the enzymatic properties of Na,K-ATPase are consistent with the view that the functional unit, in the presence of both Na^+ and K^+, is a dimer with interacting subunits.

THEORY

We consider an enzyme subunit capable of existing in three kinetically distinguishable states (Fig. 1 *a*). This would correspond to an ATPase for which the overall reaction is $ATP \rightarrow ADP + P_i$. Since here we will be concerned with initial steady-state rates, i.e., in the absence of significant concentrations of products ADP and P_i, we can put the reverse rate constants k^o_{-2} and k^o_{-3} equal to zero. For an ATPase, the state EP (Fig. 1 *a*) corresponds to the phosphorylated enzyme.

If the functional enzyme system is a dimer consisting of two identical subunits, the kinetic states for the functional units must specify the state of each subunit. The kinetic mechanism for the units, with interconnections between the states, is then as shown in Fig. 1 *b*. This rather formidable scheme can be made more transparent when the symmetry of the units is recognized: ES · EP is indistinguishable from EP · ES, etc. By grouping together in a single kinetic state corresponding symmetric substates, we obtain the simpler scheme shown in Fig. 1 *c*.

The interaction between the subunits in a dimer will generally depend on their instantaneous states. As a result the rate constants shown in Fig. 1 *c* will be different from those characterizing the single subunits. The general procedure for taking this into account has been described by Hill (9). Denoting by w_{ij} the free energy of interaction of two subunits in states i and j (relative to infinite separation of subunits), the rate constant, for example, connecting the states 12 → 22 in Fig. 1 *c*, would be

$$\lambda_1 = k_1^o \exp\left[f_{12,22}(w_{12} - w_{22})/kT\right], \qquad (1)$$

where $f_{12,22}$ is a constant associated with the pair of states considered. This corresponds to the assumption that the free energy of interaction between the transition state of subunit 1, undergoing the transition 1 → 2, and the other subunit (in state 2) is

$$(1 - f_{12,22})w_{12} + f_{12,22}w_{22}.$$

Correspondingly, the reverse rate constant is given by

$$\lambda_{-1} = k^o_{-1} \exp\left[(1 - f_{12,22})(w_{22} - w_{12})/kT\right] \qquad (2)$$

to ensure detailed balance at equilibrium (see reference 10 for details).

This kinetic model is still rather complex. It contains three different hydrolysis cycles (A–C in Fig. 1 *c*), and

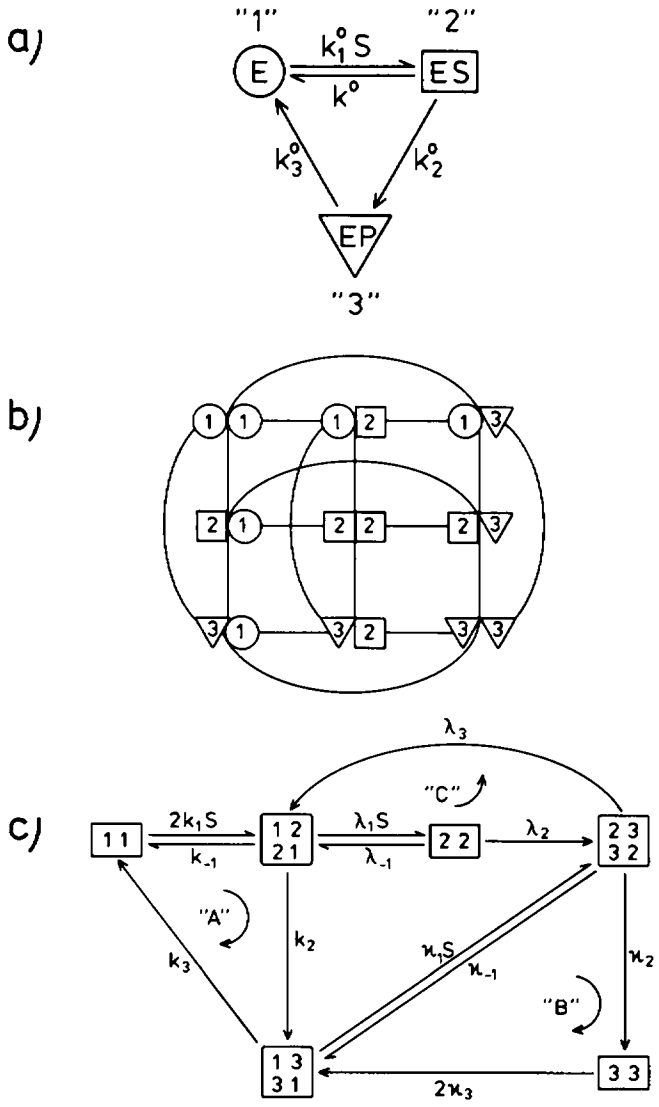

FIGURE 1 (*a*) The three states for the monomeric enzyme. (*b*) The nine-state kinetic model of a dimeric enzyme with subunit states as in *a*. (*c*) The kinetic model obtained when account is taken of the identity of symmetric dimer states. The rate constants are indicated, including the necessary statistical factors. The direction of hydrolysis is indicated by curved arrows inside the cycles denoted A, B, and C. Each square box indicates a single kinetic state of the dimer.

three steps with first order rate constants proportional to S. As a result the general steady-state rate equation contains third order terms in S. Strong negative cooperativity, however, may suppress one or the other of the cycles B and C: If binding of S to one subunit precludes the binding to the other subunit in a pair ($w_{22} \rightarrow \infty$ in Eq. 1), $\lambda_1 = 0$ and the cycle C is suppressed. The resulting model M1 is shown in Fig. 2 *a*. Alternatively, if a subunit in state 3 precludes binding of substrate to the other subunit, $\kappa_1 = 0$, cycle B is suppressed, and the scheme M2 shown in Fig. 2 *b* is obtained. Because, for Na,K-ATPase, only two experimental cycles have been observed, we confine our interest here to these two possibilities and consider them in turn below.

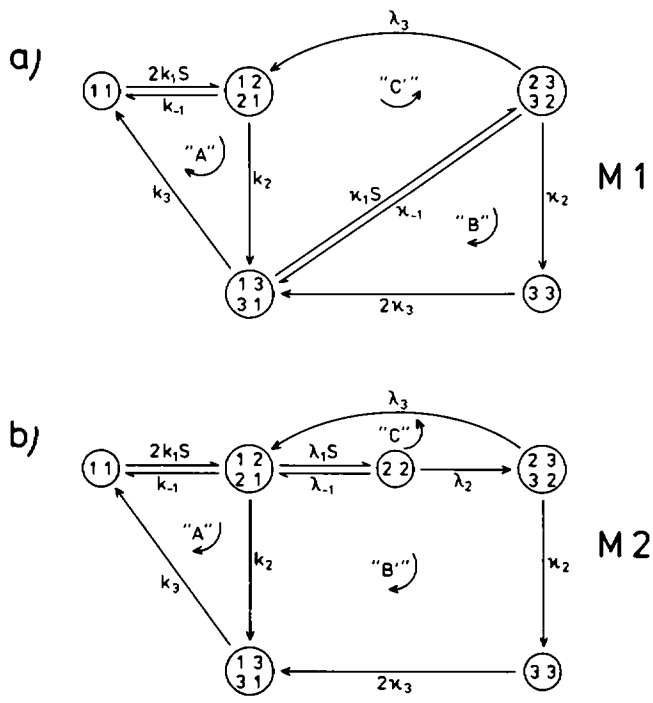

FIGURE 2 (*a*) The kinetic model M1 obtained when double substrate occupancy of the dimer is excluded. (*b*) Model M2, obtained when phosphorylation of one subunit prevents substrate binding to the adjacent subunit.

The Model M1

We first derive, in terms of the rate constants in Fig. 2 *a*, the steady-state probabilities (i.e., the relative steady-state concentrations) of the kinetic states using standard procedures (11). We obtain

$$p_{11} = N^{-1} \cdot 2k_3\kappa_3(k_{-1} + k_2)(\kappa_{-1} + \kappa_2 + \lambda_3) \tag{3}$$

$$2p_{12} = 2p_{21} = N^{-1} \cdot [4k_1k_3\kappa_3(\kappa_{-1} + \kappa_2 + \lambda_3) \cdot S + 4k_1\kappa_1\lambda_3\kappa_3S^2] \tag{4}$$

$$2p_{13} = 2p_{31} = N^{-1} \cdot 4k_1k_2\kappa_3(\kappa_{-1} + \kappa_2 + \lambda_3) \cdot S \tag{5}$$

$$2p_{23} = 2p_{32} = N^{-1} \cdot 4k_1k_2\kappa_1\kappa_3S^2 \tag{6}$$

$$p_{33} = N^{-1} \cdot 2k_1k_2\kappa_1\kappa_2S^2, \tag{7}$$

where S is the substrate concentration and N, the normalization constant, is found from $p_{11} + 2(p_{12} + p_{13} + p_{23}) + p_{33} = 1$:

$$N = 4k_1k_2k_3\kappa_1\kappa_2\kappa_3\left(R_2R_1 + R_2Z_1S + \frac{S^2}{k_3}Z_2\right) \tag{8}$$

with

$$R_1 = \frac{k_{-1} + k_2}{2k_1k_2}; \quad Z_1 = \frac{k_2 + k_3}{k_2k_3} \tag{9}$$

$$R_2 = \frac{\kappa_{-1} + \kappa_2 + \lambda_3}{\kappa_1\kappa_2}; \quad Z_2 = \frac{\lambda_3}{k_2\kappa_2} + \frac{2\kappa_3 + \kappa_2}{2\kappa_2\kappa_3}. \tag{10}$$

The steady-state rate per dimer unit is

$$v = k_3 \cdot 2p_{13} + (\kappa_2 + \lambda_3) \cdot 2p_{23} = \frac{R_2S + (S^2/k_3)(1 + \lambda_3/\kappa_2)}{R_1R_2 + R_2Z_1S + Z_2(S^2/k_3)}. \quad (11)$$

The significance of the quantities R_1, R_2, and Z_1 is as follows: At small substrate concentrations S we can ignore second order terms in Eq. 11, to obtain

$$v_A^\circ = \frac{S}{R_1 + Z_1S}, \quad (12)$$

i.e., v_A° is the steady-state rate if cycle A is the only operative cycle. This is a simple Michaelis–Menten equation, and writing this in Lineweaver–Burk form, $v_A^{\circ -1}$ vs. S^{-1}, we have

$$(v_A^\circ)^{-1} = R_1 \cdot \frac{1}{S} + Z_1. \quad (13)$$

R_1 is thus the slope, and Z_1 the ordinate intercept (multiplied by the total enzyme concentration), of such a double-reciprocal plot of the observed reaction rate. A corresponding expression, valid if cycles B and C′ are the only operative cycles, is obtained if, at sufficiently high substrate concentration, $\kappa_1 S \gg k_3$. Thus, letting $k_3 \to 0$ in Eq. 11, we obtain

$$v_B^\circ = \frac{S(1 + \lambda_3/\kappa_2)}{R_2 + Z_2S}, \quad (14)$$

and the slope and vertical intercept of a double-reciprocal plot of the pertinent data are thus

$$\text{slope} = \frac{R_2}{1 + \lambda_3/\kappa_2} \quad (15)$$

$$\text{intercept} = \frac{Z_2}{1 + \lambda_3/\kappa_2}. \quad (16)$$

If cycle B in Fig. 2 *a* is the dominant cycle (note that the order of product release is reversed in cycle *C′*, relative to B), $\lambda_3 \ll \kappa_2$, and we then have

$$\text{slope} = R_2^\circ \equiv \frac{\kappa_{-1} + \kappa_2}{\kappa_1\kappa_2} \quad (17)$$

$$\text{intercept} = Z_2^\circ \equiv \frac{2\kappa_3 + \kappa_2}{2\kappa_2\kappa_3}. \quad (18)$$

It is seen that the model M1 in principle admits the observation of two distinct "experimental" cycles, each with "its own" K_m and maximal velocity: cycle A ($K_m = R_1/Z_1$, $V_{1max} = Z_1^{-1}$) at low substrate concentration, and cycle (B + C′) [$K_m = R_2/Z_2$, $V_{2max} = Z_2^{-1}(1 + \lambda_3/\kappa_2)$] at higher substrate concentrations. The main kinetic parameters describing the entire mechanism can thus be obtained from experiments at appropriate conditions.

The total rate, v, may be written as the sum of the contributions from each of the two "experimental" cycles:

$$v_A = k_3 \cdot 2p_{13} = \frac{R_2S}{R_1R_2 + R_2Z_1S + Z_2(S^2/k_3)} \quad (19)$$

$$v_{(B+C')} = (\kappa_2 + \lambda_3) \cdot 2p_{23} = \frac{k_3^{-1} \cdot S^2(1 + \lambda_3/\kappa_2)}{R_1R_2 + R_2Z_1S + Z_2(S^2/k_3)}, \quad (20)$$

and hence the "half-of-sites index" (10) is

$$\frac{v_{(B+C')}}{v_A} = \frac{S(1 + \lambda_3/\kappa_2)}{k_3R_2}, \quad (21)$$

which increases linearly with S in analogy to the corresponding index for the two-state subunit case (10).

The rate constant k_3 is not directly obtainable from experiments, as are the parameters R and Z (with $\lambda_3 \ll \kappa_2$). But if $k_3 \ll k_2$ (and $\lambda_3 \simeq 0$) we have from Eq. 9, $Z_1 \simeq k_3^{-1}$, i.e., k_3, is the turn-over number (for cycle A), then

$$\frac{v_B}{v_A} \simeq \frac{Z_1S}{R_2}. \quad (22)$$

Under these conditions the total rate equation, Eq. 11, simplifies to

$$v = \frac{(R_2^\circ + Z_1S)S}{R_1R_2^\circ + Z_1S(R_2^\circ + Z_2^\circ S)}. \quad (23)$$

This expression involves only easily determined experimental quantities.

The main results of this analysis are then that the kinetic mechanism of a dimeric enzyme, the subunits of which may each be in three states, may give rise to only two experimentally distinct, non-overlapping hydrolysis cycles, and that the difference between rate constants characterizing chemically similar steps in the two cycles (substrate binding, product release) may be interpreted as residing in the different interactions involved between the subunits. It also leads to enhanced reactivity at larger substrate concentrations of the dimer, relative to that of the monomer, and half-of-the-sites reactivity (see below).

The explicit expressions for the rate constants and their dependence on the interaction free energies w are obtained as follows: Following Hill and Levitzki (10) we define

$$y_{ij} \equiv \exp(-w_{ij}/kT). \quad (24)$$

The rate constants k_i and κ_i are then

$$k_1 = k_1^\circ \left(\frac{y_{12}}{y_{11}}\right)^f; \quad k_{-1} = k_{-1}^\circ \left(\frac{y_{11}}{y_{12}}\right)^{1-f}$$

$$k_2 = k_2^\circ \left(\frac{y_{13}}{y_{12}}\right)^g; \qquad k_3 = k_3^\circ \left(\frac{y_{11}}{y_{13}}\right)^h \quad (25)$$

for cycle A and, analogously, for cycle B

$$\kappa_1 = k_1^\circ \left(\frac{y_{23}}{y_{13}}\right)^f; \quad \kappa_{-1} = k_{-1}^\circ \left(\frac{y_{13}}{y_{23}}\right)^{1-f}$$

$$\kappa_2 = k_2^\circ \left(\frac{y_{33}}{y_{23}}\right)^g; \quad \kappa_3 = k_3^\circ \left(\frac{y_{13}}{y_{33}}\right)^h, \tag{26}$$

where f, g, and h are constants, each "belonging" to a pair of reverse transitions, and we assume the same values for both cycles. For simplicity we assume $\lambda_2 \ll \kappa_2$ and hence ignore it below. When we introduce the following quantities:

$$y_A \equiv y_{11}/y_{12} \quad z_A \equiv y_{13}/y_{12} \tag{27}$$

$$y_B \equiv y_{13}/y_{23} \quad z_B \equiv y_{33}/y_{23} \tag{28}$$

Eqs. 24 and 25 take the simpler form

$$k_1 = k_1^\circ y_A^{-f}; \quad k_{-1} = k_{-1}^\circ y_A^{1-f}$$

$$k_2 = k_2^\circ z_A^g; \quad k_3 = k_3^\circ (y_A/z_A)_h \tag{29}$$

and

$$\kappa_1 = k_1^\circ y_B^{-f}; \quad \kappa_{-1} = k_{-1}^\circ y_B^{1-f}$$

$$\kappa_2 = k_2^\circ z_B^g; \quad \kappa_3 = k_3^\circ (y_B/z_B)_h. \tag{30}$$

If estimates of the two sets of rate constants k_i and κ_i are at hand, the magnitudes of (differences between) interaction energies may be calculated.

The Model M2

This scheme, shown in Fig. 2 *b*, is obviously more complex than model M1. However, in the simpler case in which cycle C is dominant relative to cycle B', i.e., $\kappa_2 \ll \lambda_3$, the rate expression is

$$v = \frac{R_3 S + S^2/k_2}{R_1 R_3 + S(R_3 Z_1 + Z_3(S/k_2)} \tag{31}$$

with

$$R_3 = \frac{\lambda_{-1} + 2\lambda_2}{2\lambda_1\lambda_2}; \quad Z_3 = \frac{\lambda_3 + 2\lambda_2}{2\lambda_2\lambda_3}. \tag{32}$$

Again, there are two experimental cycles, characterized by slopes R_1 and R_3 and intercepts Z_1 and Z_3, obtainable from the appropriate Lineweaver–Burk plots.

The possibility of obtaining two distinct cycles, each describable by a Michaelis–Menten equation, is lost if κ_2 is comparable to λ_3. The complete rate equation is then

$$v = \frac{R_3' S + \frac{S^2}{k_2}\left(1 + \frac{2\kappa_2}{\lambda_3}\right)}{R_1 R_3' + S\left(R_3' Z_1 + \frac{\kappa_2}{2k_1 k_2 \lambda_3}\right) + \frac{S^2}{k_2}\left[\frac{\kappa_2}{\lambda_3}\left(\frac{1}{k_2} + \frac{1}{2\kappa_3}\right) + \frac{\lambda_3 + 2\lambda_2 + \kappa_2}{2\lambda_2\lambda_3}\right]}, \tag{33}$$

where $R_3' = R_3(1 + \kappa_2/\lambda_3)$. At small substrate concentrations (S^2 terms ignored), Michaelis–Menten kinetics is obtained, but the reciprocal V_{max} is

$$V_{max}^{-1} = Z_1 + \frac{\kappa_2}{2R_3' k_1 k_2 \lambda_3}.$$

When the substrate concentration is increased, such that $k_2 \ll \lambda_1 S$, non-Michaelis–Menten kinetics is obtained.

KINETIC PROPERTIES OF Na,K-ATPase

The essential features of the minimal kinetic model for the kinetics of Na,K-ATPase referred to in the Introduction are shown in Fig. 3. Only the kinetic states necessary for a description of steady-state rate data are represented in this figure. Thus, each kinetic state in Fig. 3 represents a "pool" of intermediates in rapid equilibrium with Na^+ and/or K^+. The precise way in which these ligands should be shown to interact with the enzyme depends on whether the scheme represents the bicyclic model (full lines only) or the Albers–Post model (broken arrow included, but all steps to the right of the vertical line omitted). The direction of hydrolysis of substrate (ATP) is indicated by the curved arrows inside the cycles I and II. In cycle II the (presumed) phosphoenzyme is denoted Ex to distinguish it from EP in cycle I. The latter state consists of at least two isomeric intermediates, E_1P and E_2P, differing in conformation and in sensitivity to ADP and K^+.

The model in Fig. 3 was constructed on the basis of steady-state kinetic data in which the cycles I and II were studied separately under "Na-ATPase conditions" (cycle I, reference 1) and "Na,K-ATPase" (cycle II, reference 2) and a subsequent synthesis of the two cycles based on data for K^+ inhibition of Na-ATPase and the idea that both activities are exhibited by the same enzyme unit experiencing different conditions.

It has been possible to extract from the kinetic data all the rate constants in the model in Fig. 2 (3–5). On comparing the corresponding rate constants for the two

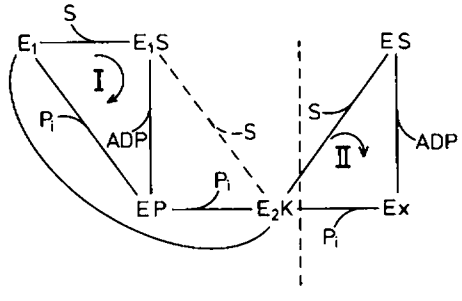

FIGURE 3 The essential features of the bicyclic model for Na,K-ATPase obtained from kinetic analyses (full lines only). The Albers–Post model is obtained when the states ES and Ex (to the right of the vertical broken line) are deleted and the broken arrow is incorporated (see Introduction). *S* denotes substrate MgATP. For both models the state EP designates at least two conformationally different, isomeric phosphoenzymes E_1P and E_2P (see text).

cycles (see Table I) rather large differences are seen: the substrate-binding rate constant is much higher in cycle I than in cycle II, while all release constants in cycle II (including that for substrate) are higher than in cycle I, indicating lower stability of the states in cycle II relative to those in cycle I. Since corresponding reactive (i.e., product releasing) species in the two cycles are similarly liganded (see references 4 and 5 for details), the kinetic differences between them must reside in the properties of the enzyme unit which, if a unit consists of a single enzyme molecule, would necessitate postulating several new conformational states.

It is clear from the analysis in the previous section that the model in Fig. 3 has properties rather similar to that of model M1, Fig. 2 *a*, and a simplified version ($\kappa_2 = 0$) of model M2 in Fig. 2 *b*: there are two distinct hydrolysis cycles, each with its own characteristic K_m, V_{max}, and slope of a double-reciprocal plot. There is a considerable enhancement of the activity at high substrate concentration (in the presence of K^+) even though the affinity of the substrate for the enzyme under these conditions (Na,K-ATPase conditions) is ~1,000 times lower than that seen in the absence of K^+ (Na-ATPase). Also, in the presence of both Na^+ and K^+, the double reciprocal plot of Na,K-ATPase activity exhibits the downwards concave curvature characteristic of negative cooperativity.

If the enzyme is a dimer and double occupancy of a unit with substrate is excluded, as in model M1, then a simple physical basis for the kinetic differences observed in the two hydrolysis cycles is evident: while both result from one subunit of a pair cycling through its (essentially only) three possible states, the different reactivities (different stabilities) of the states in the two cycles result from the different environments of the "active" subunit in the two cases. In cycle I the neighboring subunit is empty, while in cycle II it is phosphate-bound. As seen below, the differences in interaction free energies necessary to produce the striking changes in reactivity are rather modest, comparable to the stabilization free energies associated, for example, with the formation of a few hydrogen bonds (base pair formation) in nucleic acids.

TABLE I
RATE CONSTANTS DEFINED IN FIG. 2 *a* FOR Na-ATPase (CYCLE A) AND Na, K-ATPase (CYCLE B)*

Na-ATPase	Na, K-ATPase
k_1 (M^{-1} min^{-1}), 5.5×10^8	κ_t (M^{-1} min^{-1}), 6.8×10^7‡
k_{-1} (min^{-1}), 4.0×10^2	κ_{-t} (min^{-1}), 3.3×10^4‡
k_2 (min^{-1}), 2.2×10^3	κ_2 (min^{-1}), 6.0×10^4
k_3 (min^{-1}), 3.2×10^2	κ_3 (min^{-1}), 1.6×10^4

*At $[Na^+]$ = 150 mM, $[K^+]$ = 20 mM, and $[Mg^{++}]$ = 7 mM, from references 4 and 5 at T = 37°C, pH = 7.4.
‡The value from reference 5 has been multiplied by 2.5 to obtain an intrinsic value for the appropriate step in the mechanism (see reference 3 for details).

If the enzyme functions as a dimer under all conditions one would expect that Na-ATPase should exhibit the bifunctional kinetics in accordance with Fig. 2 *a*. This is not observed experimentally. In the absence of K^+ strict Michaelis–Menten kinetics are observed with high affinity substrate binding and low maximal velocity. Furthermore, at micromolar substrate concentrations, K^+ is inhibitory (when $[Mg^{++}]$ is in the millimolar range). This apparent difficulty can be resolved if, in the absence of K^+, the subunits function independently of each other, but the addition of K^+ introduces subunit interactions such that the kinetic properties (K_m and V_{max}) of ATPase in the presence, but not in the absence, of K^+ depend on the substrate concentration range, as is observed experimentally. If this is true, the addition of K^+ to the enzyme under Na-ATPase conditions, i.e., with micromolar substrate concentration, should effectively inactivate half of the subunits in the preparation due to their decreased affinity for the substrate when the adjacent units are catalytically active. Results to this effect were reported recently (Fig. 3 of reference 4). For example, at $[Na^+]$ = 100 mM, $[Mg^{++}]$ = 7 mM and in the absence of K^+, the maximal velocity for Na-ATPase was ~135 nmol ml^{-1} min^{-1}. Under the same conditions, but with $[K^+]$ = 2, 5, 10, and 20 mM, the maximal velocities were all 65–70 in the same units. This view is further supported by the experimental observation that, with Na-ATPase, >50% of the enzyme may be phosphorylated in the steady state, and by experiments on equilibrium binding of ADP in the presence and absence of K^+ by Ottolenghi and Jensen (12, 13): with no added K^+, the binding isotherms were linear in a Scatchard plot, indicating a single population of binding sites. When K^+ was present, the Scatchard plots were curved, showing negative cooperativity, but could be resolved into two straight lines, indicating two populations with different binding affinities and each with exactly half the binding capacity of the K^+-free enzyme. Furthermore, solubilization of the enzyme with the detergent $C_{12}E_8$ and use of this preparation in binding experiments in the presence of K^+ yielded linear Scatchard plots, i.e., abolishment of subunit interactions.

The choice between models M1 and M2 as representing the action of Na,K-ATPase will of course have to be made on experimental grounds. The experiments just discussed would thus tend to favor M2. But that model would have to be simplified (suppression of the B′ pathway) to comply with the observed kinetic properties, as shown in the previous section. This simplification would occur if the ADP-release rate constant κ_2 is much smaller than the dephosphorylation constant λ_3 and, at least for Na-ATPase, the reverse is true (see Table I).

There is, however, kinetic evidence favoring model M1. Thus, Askari and Huang (14, 15) found that the rate of dephosphorylation of phosphoenzyme formed from enzyme + inorganic phosphate (in the presence of Mg^{++}) was decreased by the addition of ATP, indicating that

ATP binds to the phosphoenzyme. K^+ did not affect this effect of ATP. Similarly, Fukushima et al. (16) observed the same effect of ATP on phosphoenzyme formed by enzyme + ATP. Furthermore, Nørby et al. (17), in an analysis of transient kinetic experiments on the dephosphorylation in the presence of K^+ of phosphoenzyme formed under Na-ATPase conditions, found that K^+ decreased the rate of disappearance of E_1P (the first in a sequence of three kinetically isomeric phosphoenzyme forms found in Na-ATPase). The simplest explanation of this effect is that K^+ binds to E_1P in such a way that the resulting adduct neither dephosphorylates nor is converted to the succeeding phosphoenzyme form.

These considerations suggest that the form "E_2K" in Fig. 3, the enzyme form to which substrate adds in cycle II, is kinetically equivalent to, and may in fact be, a semiphosphorylated dimer and thus should be grouped together with EP in Fig. 3. If this is done the resulting scheme is identical in form to that shown as model M1 in Fig. 2 *a* (with $\lambda_3 = 0$).

Interaction Free Energies

If it is accepted that, in the presence of K^+, Na,K-ATPase is a functional dimer according to model M1 in Fig. 2 *a*, then an estimate of the differences in interaction free energies necessary to produce the observed kinetic differences can be computed from the known rate constants (3–5) through the use of Eqs. 29 and 30. To illustrate typical orders of magnitude, we assume that, in cycle I, the interaction energy between two subunits, one of which is empty, is independent of the nature of the ligand on the adjacent subunit, leading to $y_A = z_A = 1$. It follows that $k_i = k_i^o$ (Eq. 29). Using appropriate values of rate constants from Table I and Eq. 30 we then obtain

$$y_B^{-f} = \frac{\kappa_1}{k_1^o} = 0.124 \tag{34}$$

$$y_B^{1-f} = \frac{\kappa_{-1}}{k_{-1}^o} = 82.5, \tag{35}$$

and hence

$$y_B = \frac{82.5}{0.124} = 665, \tag{36}$$

and, from Eq. 34,

$$f = 0.32. \tag{37}$$

Assuming g (Eq. 29) to be 0.8, as an example, we obtain

$$z_B^{0.8} = \frac{\kappa_2}{k_2^o} = 27.3, \tag{38}$$

leading to

$$z_B = 62.4. \tag{39}$$

Finally, h is calculated from

$$\left(\frac{y_B}{z_B}\right)^h = \frac{\kappa_3}{k_3^o} = 25, \tag{40}$$

yielding

$$h = 1.36. \tag{41}$$

Using the defining equations for y_B and z_B (Eqs. 28) we now have

$$\exp\left[(w_{23} - w_{13})/kT\right] = 665, \tag{42}$$

from which

$$w_{23} - w_{13} = RT\ln 665 = 16.7 \text{ kJ/mol}, \tag{43}$$

i.e., (compare Figs. 3 and 1 *c*), the state EP · ES is more unstable than the state EP · E by ~17 kJ/mol. Likewise,

$$\exp\left[(w_{23} - w_{33})/kT\right] = 62.4, \tag{44}$$

leading to

$$w_{23} - w_{33} = 10.6 \text{ kJ/mol} \tag{45}$$

and

$$w_{33} - w_{13} = 6.1 \text{ kJ/mol} \tag{46}$$

as the "destabilization free energy" of the diphosphorylated dimer relative to the monophosphorylated one. It is perhaps suggestive that the magnitudes obtained for the free energy differences are as those associated with the formation of a single base pair in double-helix stabilization of single-stranded nucleic acids (18). This could mean that the different interactions may be manifested through a change in the intra- or intersubunit hydrogen bond structure of the dimeric protein unit.

DISCUSSION

In the Introduction two serious objections against the Albers–Post model were reiterated. Further support for the distinction between hydrolysis cycles in the two activities may be derived from the recent work of Huang et al. (24) on the effect on the kinetic properties of Na,K-ATPase of the detergent $C_{12}E_8$, at concentrations less than that yielding solubilization. Their kinetic data for Na,K-ATPase and Na-ATPase (Figs. 3 and 6, respectively, of reference 24) are consistent with a three-state model in which $C_{12}E_8$ binds to all kinetic species for Na-ATPase, resulting in noncompetitive inhibition, whereas for Na,K-ATPase only the empty enzyme form binds $C_{12}E_8$, yielding activation at subsaturating substrate concentrations. Hence at most one of the kinetic intermediates of the Na-ATPase cycle can be involved in the Na,K-ATPase hydrolysis.

These results, together with those of Nørby et al. (17) discussed in the previous section, that K^+ may bind to E_1P, the first of several phosphoenzyme intermediates formed in the Na-ATPase cycle without leading to its rapid removal

(by dephosphorylation or conversion to the subsequent phosphoenzyme form), suggest a new interpretation of the enzyme form to which the substrate adds in Na,K-ATPase. If, in the presence of K^+, the enzyme is a functional dimer, the E_1P state may be identified with a semiphosphorylated dimer which, with K^+ bound in the above manner, can receive a new substrate molecule on the empty subunit. In terms of a dimeric enzyme these considerations lead to the model shown in Fig. 4. As a kinetic scheme involving the states in rectangular boxes it is identical in form and properties to the model M1 discussed above. With respect to the interpretation of the substrate binding enzyme form in the Na,K-enzyme it is an amendment of the previously published bicyclic scheme (2–5). With this interpretation the Na-ATPase cycle, cycle A in Fig. 4, is not merely an irrelevant sidepath at physiological concentrations in the intact transport system, but a necessary prerequisite for the attainment of the enhanced reactivity apparently necessary in a cell. In essence this cycle is necessary to "prime" the enzyme for Na-K exchange.

The substrate curve corresponding to the model in Fig. 4 will exhibit negative cooperactivity. This is most easily seen from the "half-of-sites" index, Eq. 21 (with $\lambda_3 = 0$):

$$\frac{v_B}{v_A} = \frac{S}{k_3 R_2}. \tag{21a}$$

Using the published values for R_2 (5), corresponding to $[Na^+] = 150$ mM and $[K^+] = 20$ mM, $R_2 = 6.6 \cdot 10^{-2} \cdot 0.66\ \mu M \cdot$ min, and k_3 (Table I), we have

$$\frac{v_B}{v_A} = \frac{S}{14}\ (S \text{ in } \mu M), \tag{47}$$

and thus, for "Na-ATPase conditions" ($S = 1\ \mu M$) and

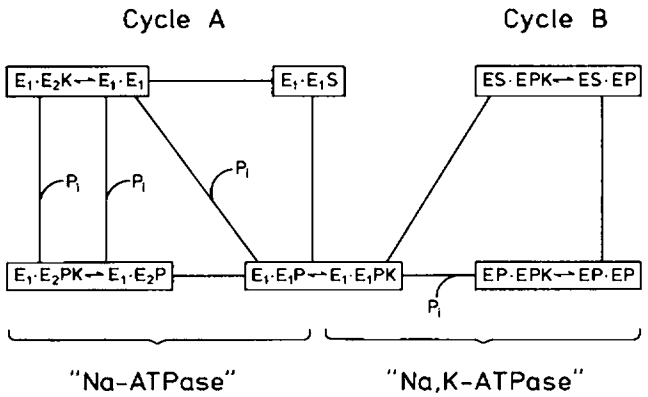

FIGURE 4 The model proposed for the hydrolytic action of Na,K-ATPase, based on a dimeric enzyme, according to model M1 in Fig. 2 *a*. Earlier kinetic results have been incorporated. Rapid equilibrium with cation binding (within boxes) is assumed. Na^+ and Mg^{++} are assumed present, but are not explicitly accounted for in the scheme (see references 5 and 6 for a detailed analysis of the kinetic effects of K^+ and Na^+). Na-ATPase and Na,K-ATPase indicate the conditions (see text) necessary for the experimental observation of the two parts of the scheme.

"Na,K-ATPase conditions" ($S = 2$ mM) we obtain:

$$\frac{v_B}{v_A}(\text{Na-ATPase}) = 7 \cdot 10^{-2} \tag{48}$$

$$\frac{v_B}{v_A}(\text{Na,K-ATPase}) = 143, \tag{49}$$

reflecting the fact that as the substrate concentration increases, at steady state more enzyme will be found distributed among the intermediates of cycle B. A substrate curve exhibiting these features has been published (25).

During the last ten years a number of techniques have been used to investigate whether Na,K-ATPase is, or is not, a functional dimer.[1] These include the demonstration of apparent "half-of-the-sites" reactivity in phosphorylation experiments (26), when Ca is substituted for Mg, and in cross-linking experiments (27), as well as ligand-induced cooperactivity under equilibrium conditions (12, 13, 15, 16). This evidence has been reviewed by Glynn (6) and by Askari and Huang (28). More recent results by Hayashi et al. (29) on solubilized Na,K-ATPase, by Jensen and Ottolenghi (30), using radiation inactivation, and by Hebert et al. (31) and Ovchinnikov et al. (32), on determinations of the three-dimensional structures of Na, K-ATPase, which show that the unit cell consists of two $\alpha\beta$ protomers, are all consistent with the view that Na,K-ATPase in the membrane is a functional dimer. The findings that solubilized enzyme preparations, appearing monomeric by sedimentation velocity measurements (33), cross-linking analysis (34, 35), or gel filtration (36), possess enzymatic activity are not inconsistent with this view particularly since uncertainties remain as to whether the enzyme in such preparations is monomeric also under assay conditions.

There is evidence that other membrane-bound ATP-hydrolyzing enzymes are of a dimeric nature. Thus, radiation inactivation experiments on Ca-ATPase from sarcoplasmic reticulum (37) result in a target size of 210,000–250,000 D, corresponding to two protomer units. This enzyme also displays complex substrate kinetics, exhibiting apparent negative cooperactivity (i.e., a downwards concave Lineweaver–Burk plot) (38, 39). Various models involving a dimeric enzyme have been proposed (40–43) (see also the review by Inesi and de Meis [44]). It has been shown that ATP decreases the release of vanadate (a phosphate analogue) from vanadate-bound enzyme (45). This is analogous to previously mentioned experiments with Na,K-ATPase (14, 15) and indicates the binding of ATP to the phosphoenzyme. This suggests that the kinetics of Ca-ATPase can be analyzed in accordance with the

[1]By "subunit" or "monomer" in the context of this paper is meant an $\alpha\beta$ protomer; α is a large polypeptide ($M_r \sim 95{,}000$) that contains the catalytic site, whereas β ($M_r \sim 45{,}000$) is a glycoprotein with unknown function.

procedures described herein. Thus, for example, we can calculate the "half-of-sites" index v_B/v_A using published values of pertinent kinetic parameters (38; Table I). If we use the approximate expression, Eq. 22, we obtain

$$\frac{v_B}{v_A} = \frac{S \cdot Z_1}{R_2} = \frac{S \cdot V'_{max}}{V_{max} \cdot K_{0.5}}, \quad (50)$$

where the last equation is obtained using the definitions of Z_1 and R_2 in terms of the nomenclature in reference 38. At 1 μM Ca^{+2} we obtain, for S = 10 μM and 5 mM, respectively,

$$\frac{v_B}{v_A} = \frac{10}{235} = 4.3 \cdot 10^{-2}$$

$$\frac{v_B}{v_A} = \frac{5 \cdot 10^3}{235} = 21.3,$$

and, for the same substrate concentrations, but $[Ca^{++}]$ = 100 μM,

$$\frac{v_B}{v_A} = \frac{S}{555}$$

$$= 1.8 \cdot 10^{-2} \quad \text{at } S = 10\ \mu M$$

$$= 9 \quad \text{at } S = 5\ mM.$$

During the last few years evidence has been accumulated pointing to the possibility that the enzyme H^+-ATPase from plasma membranes of the fungus *Neurospora crassa* is a functional dimer with apparently positive cooperativity (46, and references therein). If so, its kinetic behavior may be analyzed along the same lines. Similar remarks apply to gastric K,H-ATPase (47).

In many instances the cooperative substrate curves obtained with Na,K-ATPase or Ca-ATPase have been ascribed to a regulatory, as distinct from catalytic, action of the substrate ATP. In no case, however, has a specific model incorporating such regulatory action been analyzed and shown to be consistent with the observed data. In contrast, the dimeric models described here lead directly to the observed cooperative kinetics and, in addition, provide a plausible physical interpretation of states and transitions that may be subjected to experimental verification.

On the other hand, the existence of two distinct, doubly connected, cycles provides the system with regulatory power: the substrate level and the ionic concentrations (through their influence on the rate constants) determine to what extent one or the other cycle is used (see Eq. 21. This situation has features in common with the phosphorylatable enzymes (e.g., phosphorylase, glycogen synthase), in which phosphorylation–dephosphorylation is a major regulatory mechanism, as surveyed recently by Krebs (48).

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