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The application of vectorial coupling theory to the calcium pump

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Abstract

Models for the calcium pump of the E_1E_2 type appear to be inconsistent with new evidence for the binding of internal and external calcium ions, simultaneously, to the free pump. The models are shown here to be incomplete but not necessarily wrong; they omit the required modulation of the mobility, specificity, and enzyme activity of the pump, which is brought about through substrate-controlled conformational changes. A reaction scheme based on the E_1E_2 model but incorporating the conformational equilibria in question is shown to account for a variety of experimental findings, including those at odds with the simple model: (i) binding of lumenal Ca^{2+} to the free as well as the phosphorylated pump; (ii) uncoupled exit of lumenal Ca^{2+} at high concentrations; (iii) the absence of any effect of lumenal Ca^{2+} on the binding of external Ca^{2+}; (iv) uncoupled ATPase activity in dimethyl sulfoxide.

Key words: ATPase; Active transport; Coupling mechanism; Uncoupled transport; Transport kinetics; Binding force

1. Introduction

Two main enzyme conformations are represented in an E_1E_2 model of the calcium pump [1–4]: outward-facing, with a calcium binding site exposed on the cytoplasmic side of the membrane (E_1), and inward-facing, with the calcium site exposed on the lumenal side of the membrane (E_2). The pump cycles between these states, bearing calcium ions across the membrane in one half of the cycle and returning empty in the other half. The substrates (the driven substrate, Ca^{2+}, and the driving substrate, ATP and its hydrolysis products) add in a fixed order, with a different order on the two sides – Ca^{2+} adds first to the outward-facing pump, followed by ATP, whereas inorganic phosphate adds first to the inward-facing pump, followed by Ca^{2+}. Ca^{2+} binds with high affinity outside and with low affinity inside, which facilitates the loading and unloading of the carrier as the substrate is transferred from a region of low to a region of high concentration.

While the additional conformational states detected in the course of the transport reaction are easily accommodated by the model, other findings do not seem to fit. Internal Ca^{2+} adds directly to the free as well as to the phosphorylated form of the pump and when bound inside in the absence of inorganic phosphate has no effect on the binding of external Ca^{2+} [1,5,6]. Ca^{2+}, it appears, adds on both sides at the same time, rather than on one side or the other. The issue is central, inasmuch as transport mechanisms fall into two distinct classes: those in which a substrate binding site cycles between inward-facing and outward-facing states, and those in which the substrate moves between binding sites fixed on opposite sides of the membrane. The structures of the membrane proteins involved would differ in a fundamental way, and so would the properties of the system.

Before the E_1E_2 model is abandoned, in the face of the new evidence, its full implications need to be examined. A problem arises because the usual models for active transport, either primary or secondary, are incomplete [7]. Conventionally, the ordinary carrier model, originally proposed to explain passive transport of a single substrate [8], is extended to cover the case of co-transport by allowing two substrates to add to the carrier to form a ternary complex that is translocated across the membrane, as in Fig. 1. For movement of the two substrates to be linked, the binary complex with either substrate alone must be immobile (other-

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wise the system would degenerate into a facilitated, that is, an uncoupled, system, carrying one substrate across the membrane without the other). Implicit in this model, which can represent either co-transport or an ATP-driven pump [9–12], is the transformation of the mobile free carrier into an immobile complex with the first substrate, or, where the order of substrate addition is fixed, the transformation of a free carrier having a site for only one of the substrates, into a binary complex having sites for both. These transformations are postulated but not accounted for, though a ligand-induced conformational change is a familiar concept, the basis of allosteric enzyme theory and of cooperativity in substrate or modifier binding: the idea is that two conformations of an enzyme exist, in equilibrium, and that the substrate necessarily shifts the equilibrium to favour the form to which it is more strongly bound; if the affinity is far higher for the less stable conformation, the shift can be abrupt.

New properties emerge once these conformational changes are made explicit [7]. When a scheme of minimal complexity for the substrate-controlled interconversion of carrier conformations – conformations that are mobile or immobile, have sites for one or both substrates, or have altered enzyme activity – is incorporated into a scheme for co-transport or ATP-driven transport, the expanded scheme is found to explain a variety of observations, including those at odds with the simple E_1E_2 model.

2. Vectorial coupling theory

In a coupled vectorial process the uncoupled reaction (slippage), in which either reaction proceeds independently, cannot be avoided altogether [7,13]. This is because the reaction branches into coupled and uncoupled paths, with the traffic controlled by the carrier conformations along the way. The changing conformations depend on specific interactions with substrates: a substrate may either increase the *rate* at which one conformation is converted to another, by becoming more strongly bound in the transition state; or it may shift the *equilibrium* between two conformations, by binding more strongly to one than the other. The increased strength of the substrate binding forces in successive carrier states determines, respectively, the speed, or the abruptness, of the conformational change. Consequently the tightness of coupling, i.e., the ratio of coupled to uncoupled reaction rates, is related to the increase in the binding force; and because, for the sake of efficiency, coupling has to be tight, a large increase in the binding energy in successive carrier states is called for, which has implications for the structures of the carrier complexes formed.

2.1. Vectorial catalysis

In a symmetrical ordered mechanism (Fig. 1), where the binary complex with S is immobile and the ternary complex with both substrates is mobile, the ratio of coupled to uncoupled rates is found to depend on the increased binding force in the transition state:

$$\bar{V}_{ST_o}/\bar{V}_{S_o} < K_{T_o}/K_T^{ts} \quad (1)$$

$\bar{V}_{ST_o}$ and $\bar{V}_{S_o}$ are the maximum rates of coupled and uncoupled transport, respectively, K_{T_o} is the dissociation constant for substrate T in the external medium, and K_T^{ts} is the virtual substrate dissociation constant in the transition state (this relationship was derived earlier – see Ref. [7], Eq. (10)). The affinity of T in the transition state is seen to be higher than the apparent affinity by a factor greater than the coupling ratio, $\bar{V}_{ST_o}/\bar{V}_{S_o}$.

2.2. Induction of a conformational change: the ordered addition of substrates

In the scheme in Fig. 1, S, adding first, forms a binary complex that is immobile – unlike the free carrier and the ternary complex – and in which a binding site for the second substrate, T, is exposed. To account for the behaviour, two different carrier conformations are postulated, as in Fig. 2. The reasoning is as follows. Since only one of the substrates can add to the free carrier, a conformation exists containing a site for this substrate alone (C′); and since at a later stage of the reaction both substrates are bound, another conformation exists in which both sites are exposed (C″). The one-site conformation C′ and the two-site conformation C″ will be in equilibrium, though one may be

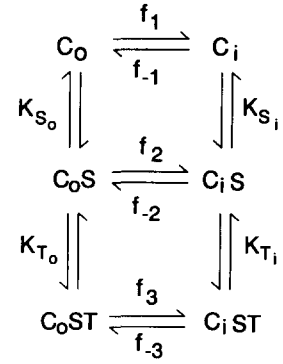

Fig. 1. Carrier scheme for co-transport: a symmetrical ordered mechanism. The order of addition of substrates to the carrier is the same on the two sides of the membrane – S first and T second. The carrier alternates between two conformations, C_o and C_i, in which substrate sites face outward and inward, respectively; substrates in the outer or inner compartment add to these, forming an outward-facing or inward-facing complex. For the transport of the substrates to be coupled, C_oS and C_iS must be immobile (f_2, $f_{-2} \ll f_1$, f_{-1}, f_3, f_{-3}).

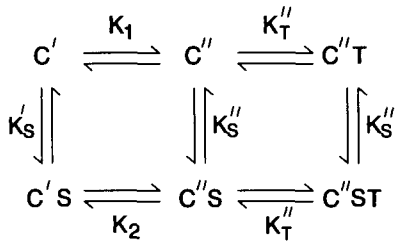

Fig. 2. Reaction scheme accounting for an ordered addition of substrates – S first and T second. The carrier exists in two conformations: C′, with a site for S but none for T, and C″, with sites for both. For a strictly ordered mechanism and for tight coupling (see Ref. [7]), the free carrier is predominantly in conformation C′ ($K_1 = [C']/[C''] \gg 1$) and, in the symmetrical ordered mechanism, the complex with S is predominantly in conformation C″ ($K_2 = [C'S]/[C''S] \ll 1$). Substrate T can add to the two-site conformation of the free carrier, forming C″T, which is expected to be fully mobile (see text).

favoured; and a substrate molecule induces a conformational change by binding preferentially to one. To ensure that S binds first, the free carrier is overwhelmingly in the mobile conformation C′ ($K_1 = [C']/[C''] \gg 1$), while the complex with S is in the immobile conformation, C″S ($K_2 = [C'S]/[C''S] \ll 1$). Uncoupled transport cannot be completely eliminated because each substrate, alone, forms a mobile as well as an immobile complex: C′S should be mobile because C′ is the mobile carrier conformation, and C″T should be mobile because T catalyses carrier movement when it adds to C″S (see Fig. 1) and it should do so when it adds to C″. The concentrations of these forms determine the uncoupled rates, while the concentration of C″ST determines the coupled rate. The ratio of coupled to uncoupled flux for substrate T, $\bar{v}_{ST}/\bar{v}_T$, is found to be related to the ratio of dissociation constants in the initial complex with S and in the tight complex to which it is converted (Eqn. 17, reference 7):

$$\bar{v}_{ST}/\bar{v}_T \ll K'_S/K''_S \quad (2)$$

(where v is the rate measured at a less-than-saturating concentration of substrate T). The important result here is that K'_S/K''_S is much larger than the coupling ratio, $\bar{v}_{ST}/\bar{v}_T$. Binding in C″S, therefore, has to be very much stronger than in the initial complex.

2.3. The coupling of binding energies and the coupling of vectorial processes

Substrate binding energy can be used to two different ends: one, tight coupling of a vectorial process to a chemical reaction or to another vectorial process, as described above; the other, rapid turnover of the system under physiological conditions, as discussed by Jencks [1]. The mechanisms are closely related, both dependent on induced conformational changes. Rapid turnover is achieved through the avoidance of high-energy or low-energy intermediates, making the rates of individual steps similar. An example is provided by offsetting binding energies in the calcium complex of the phosphorylated pump [14]: in the outward-facing pump, calcium ions are tightly bound and the phosphate bond is relatively unstable ('high-energy'); in the inward-facing pump, calcium ions are loosely bound and the phosphate bond is stable ('low-energy'). The arrangement makes for efficient pumping since calcium is to be taken up at a low concentration outside and expelled into a solution containing a high concentration inside.

The association of strong binding of one substrate with weak binding of another does not imply that the affinity of one is enhanced at the expense of the other. As the calcium binding sites in the pump are distant from the ATPase active centre [15–17] any adjustment of binding strengths or other properties must be communicated from site to site by means of conformational changes. The conformation assumed by the transport protein is determined by a balance of energies within the folded protein structure, which can be disturbed by the forces of interaction with a bound substrate molecule or ion. Weber [18] has investigated the problem of linked interaction energies, in particular of cooperativity and anti-cooperativity in the binding of small molecules to proteins; linkage depends on preferential ligand binding to one conformational state of the protein, which shifts the equilibrium to favour that form and as a result alters the affinity of a second ligand binding preferentially to the same or another form of the protein.

Substrate binding energy, then, is coupled directly to 'binding energy' within the polypeptide chain and thence to conformational changes, but only indirectly to the binding of other substrates. The binding constants for two substrates are linked only insofar as there is an inescapable relationship, governed by the principle of microscopic reversibility, among *all* the constants in the cyclic transport process. In the scheme in Fig. 3 (an asymmetrical ordered reaction, like the E_1E_2 model) weaker binding of substrate S inside than outside ($K_{S_i} > K_{S_o}$) is necessarily compensated by adjustments in other constants, such that the following equality is maintained:

$$K_{S_o}K_{T_o}f_1/f_{-1} = K_{S_i}K_{T_i}f_3/f_{-3} \quad (3)$$

Given that the dissociation constants of two substrates can be set independently, there can be a kinetic advantage in pairing strong and weak binding. Consider the case where, to facilitate unloading, the affinity of the driven substrate S is weak inside ($K_{S_i} \gg K_{S_o}$), while the driving substrate T has the same affinity inside and outside ($K_{T_i} = K_{T_o}$). Eq. (3) requires that $f_{-1}/f_1 \ll 1$ or $f_3/f_{-3} \ll 1$, and as a result entry, which depends on f_{-1} and f_3, may be retarded. But low values of f_{-1} and f_3 can be avoided if the affinity of

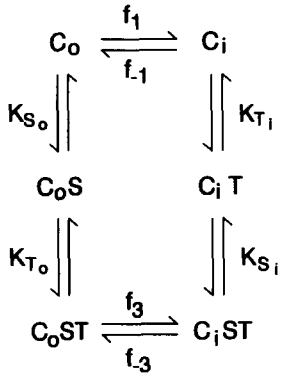

Fig. 3. Asymmetrical ordered co-transport mechanism. The order of substrate addition is different on the two sides of the membrane: S first and T second on the outside, T first and S second on the inside. The binary complex of each substrate fails to move across the membrane because the corresponding complex is missing on the opposite side.

the driving substrate T is high inside, to balance the low affinity of S ($K_{T_i} \ll K_{T_o}$); high affinity of internal T may be acceptable because its concentration, relative to that at equilibrium, is necessarily maintained at a low level. This, presumably, is the reason for offsetting binding energies in the calcium pump.

3. The calcium pump

According to the E_1E_2 model the order of substrate addition is different on the two sides of the membrane. On the cytoplasmic side (outside) Ca^{2+} adds to the free carrier to form an immobile complex, which on reaction with ATP is converted to a phosphoryl-carrier derivative that is mobile; on the lumenal side (inside), inorganic phosphate reacts with the free carrier to form an immobile phosphoryl derivative, which on addition of Ca^{2+} becomes mobile [1–4,19]. The reaction corresponds to the asymmetrical ordered scheme in Fig. 3, not the symmetrical scheme in Fig. 1, which has slightly different properties. In an asymmetrical mechanism, a binary complex, for example that on the outside (C_oS), fails to move across the membrane because

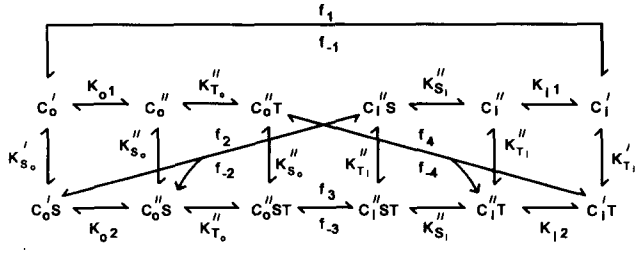

Fig. 4. Expanded scheme for an asymmetrical ordered co-transport mechanism (S followed by T outside, T followed by S inside). The scheme is derived from the simple model in Fig. 3 by allowing for a substrate-induced conformational change, as in Fig. 2.

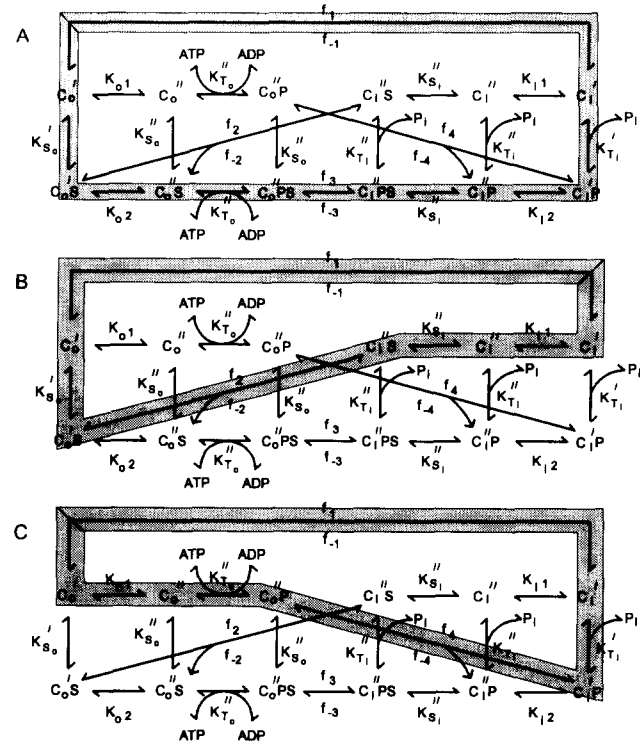

Fig. 5. Expanded reaction scheme for the calcium pump (an asymmetrical ordered mechanism, as in Figs. 3 and 4). S represents 2 Ca^{2+}; 'outside' (subscript o) refers to the cytoplasmic side of the membrane, 'inside' (subscript i) to the lumenal side. (A) The path for coupled transport. (B) The path for uncoupled transport of Ca^{2+}. (C) The path for uncoupled hydrolysis of ATP.

the corresponding complex on the other side (C_iS) does not exist (or exists at an extremely low concentration), and not necessarily because the carrier conformation is inherently immobile. All carrier species, therefore, can be assumed to be capable of movement [7]. It follows that a binary complex of T, if formed on the outside (C_oT), will be mobile, because the corresponding complex on the other side of the membrane (C_iT), being a normal intermediate in the transport cycle, is readily formed. Another consequence is that the binary complex need not be overwhelmingly in the two-site conformation, so that $K_2 = [C'S]/[C''S] \leq 1$, which slightly alters the relationship in Eq. (2) (Eq. (21), Ref. [7]):

$$\bar{v}_{ST}/\bar{v}_T < K'_S/K''_S \quad (4)$$

Incorporating the scheme in Fig. 2 for a substrate-induced conformational change into the transport scheme in Fig. 3 produces the enlarged scheme in Fig. 4. The corresponding scheme for the calcium pump is shown in Fig. 5 (where S = 2 Ca^{2+}).

3.1. Uncoupled exit of lumenal Ca^{2+}

In an asymmetrical ordered mechanism, the second substrate forms a mobile complex with the two-site

conformation of the free carrier (C″T, Fig. 2). At a sufficiently high concentration of T (in the absence of the first substrate S), the carrier is entirely converted to this complex, and slippage should approach the maximum rate of coupled transport. This may explain observations on the calcium pump reported by De Meis and co-workers: in the absence of ATP, ADP or P_i, Ca^{2+} rapidly leaks out of vesicles loaded with a high calcium concentration [20]. The rate is about the same as that of coupled exit. The model (Fig. 5B) predicts that internal calcium, because it adds second on the inside, should be able to form a mobile complex with the free carrier at high concentrations. The resulting uncoupled exit should be inhibited by calcium and phosphate in the outside medium: calcium, adding first to the outward-facing carrier, should convert the carrier to an immobile form; and phosphate, adding first to the inward-facing carrier, should prevent internal calcium from forming the mobile binary complex in the uncoupled pathway. The inhibition of exit by phosphate should be reversed by ADP, with ensuing synthesis of ATP, because under these conditions exit proceeds through the coupled pathway. The system does behave in this way. It is likely, therefore, that the leak is not through a simple channel but through the pump, working in the uncoupled mode.

3.2. The order of substrate addition inside

In a perfectly ordered mechanism a saturating concentration of the substrate that adds second draws all the carrier into the form of the ternary complex, even at a relatively low, non-saturating concentration of the first substrate; in a perfectly random mechanism, in which either substrate can add to the free carrier, the binding of one substrate is unaffected by the other. This principle was made use of by Jencks and coworkers [1,5] to decide whether the mechanism of the calcium pump is really ordered. In the E_1E_2 model, inorganic phosphate (P_i) adds to the carrier and reacts to form the phosphorylated derivative before lumenal Ca^{2+} can add. Lumenal calcium, therefore, at a sufficiently high concentration, should draw all the carrier into the phosphorylated form, even where the reaction would be incomplete in its absence. In the experimental test, the enzyme was incubated with P_i, at a fixed concentration of Mg^{2+}, in the presence of increasing concentrations of lumenal Ca^{2+}, beginning with zero. The result, contrary to expectation, was that though calcium increased the concentration of the phosphoryl derivative it failed to force all the carrier into this form at subsaturating concentrations of Mg^{2+}. It was therefore concluded that both the free carrier and the phosphorylated derivative contain a binding site for Ca^{2+}, in disagreement with the E_1E_2 model. But, as was seen above, the complete model does allow internal (i.e., lumenal) Ca^{2+} to bind to the free carrier (Fig. 5B). The two findings – binding inside, and the passive leak – are mutually supportive, and consistent with the E_1E_2 model. But the findings do require some adjustment of the model in its simplest form: substrate addition inside is only partially ordered. The dissociation constant for binding of internal calcium to the phosphorylated pump is estimated to be about 1 mM, and for binding to the free pump about 5–6 mM [5]. The preference for ordered addition is lower than expected.

3.3. Evidence for a bilateral transport model (a model in which substrate binding sites are simultaneously exposed on both sides of the membrane)

To distinguish between a mechanism of the E_1E_2 type, in which calcium binding sites alternately face inward and outward, and a mechanism in which the sites are simultaneously accessible to substrates in the solutions on either side of the membrane, Myung and Jencks [21] measured the binding of external calcium, with either empty vesicles or vesicles loaded with a high concentration of Ca^{2+} (30 mM). Binding was estimated by reaction of the enzyme with labelled ATP, following a brief period of equilibration (10 s) with external Ca^{2+}. As inner and outer forms of the free carrier (C'_i and C'_o in Fig. 5) should be in rapid equilibrium (since a kinetically significant step corresponding to their interconversion cannot be detected [4]), there should have been an instantaneous competition between inner and outer Ca^{2+} for the carrier. Instead, neither the binding curve for external calcium nor the amount of calcium bound outside was affected by internal calcium.

Though not predicted by the simple E_1E_2 model, the observations can be explained by the expanded scheme. Under the conditions of the experiment, where phosphorylated forms of the pump can be ignored (ATP enters only in the assay for the external calcium complex), the reaction may be represented by the uncoupled path traced out in Fig. 5B. One assumption is necessary: the rate limitation in uncoupled exit should be the conformational change from C'_i to C''_i rather than the conformational change in which the carrier moves from inward-facing to outward-facing, C''_iS to C'_oS. This condition seems reasonable, since (i) the equilibrium of C'_i and C''_i favours the former (see above) and is not a step involved in coupled transport; (ii) carrier reorientation is likely to be fast, judging by the fact, as noted above, that no kinetically significant step corresponding to this conformational change can be detected in coupled transport. At equilibrium with internal and external Ca^{2+}, the complex will be a mixture of internal and external forms, the proportion being dependent on f_2/f_{-2}. The equilibrium is likely

to favour the external forms, in view of the much higher substrate affinity outside than inside; that is, conversion of an inner to an outer complex involves a strengthening of substrate binding, so that the conformational change is driven by substrate binding forces. Whatever the proportion of internal and external complexes at equilibrium, the complex formed with internal calcium will be available on the outer surface of the membrane. And when the concentration of external calcium is reduced, the steady-state level of the inner complex $C_i''S$ will remain low if the rate of its formation from internal Ca^{2+} is much lower than the rate of its conversion to the outer complex $C_o'S$. The mechanism has the desired property of making the complex formed at equilibrium with internal calcium available outside, while isolating events at the external surface from the internal solution under steady-state conditions. The complex formed with internal Ca^{2+} should therefore be able to enter into the equilibrium with external calcium, contributing, equally with the free carrier, to formation of the external calcium complex that in the experiment is assayed by reaction with ATP. It follows that even saturating concentrations of internal calcium should have no effect on the binding curve for external calcium, as observed.

Another suggestive finding, reported by Meszaros and Bak [22], was the absence of a lag phase in the uptake of Ca^{2+} after addition of ATP to the external calcium complex, as if external calcium could add to the complex containing internalized calcium. However, according to Jencks et al. [5], the expected half-time for the lag phase, about 5 ms, may be too short to be detected in these experiments. In another study, also by Meszaros and Bak [6], empty or calcium-loaded vesicles were exposed to external calcium for 30 s before addition of ATP. The total amount of phosphorylated enzyme produced after 50–100 ms was found to be unaffected by internal calcium (5 mM), though the rate of its formation appeared to be somewhat reduced. The mechanism given above suggests that both should have been independent of internal Ca^{2+}. The rate estimates may be open to question, however: (i) the earliest measurements were made after 30 ms, close to the time of the maximum; (ii) in the similar experiments described above [21], where the phosphorylated enzyme was assayed after 13 ms, internal Ca^{2+} had no effect.

3.4. Uncoupled ATPase activity in dimethyl sulfoxide

A shift in K_{o1} allows the second substrate in the ordered mechanism, ATP, to form a mobile complex with the outward-facing free carrier, which in this case is a phosphoryl-carrier derivative. The resulting uncoupled cycling allows ATP to be hydrolysed in the absence of Ca^{2+} (Fig. 5C). In dimethyl sulfoxide solutions, in the absence of calcium, the system has been observed to gain ATPase activity, and the mechanism appears to be that of the normal pump since a phosphorylated carrier derivative is formed [23]. With furylacryloyl phosphate instead of ATP as the substrate the rate of hydrolysis in dimethyl sulfoxide is high [24]. One interpretation is that the specificity of the inward-facing free carrier, which normally reacts with inorganic phosphate, has been broadened, allowing ATP or furylacryloyl phosphate to react, inappropriately; another is that the equilibrium governed by K_{o1} is displaced in dimethyl sulfoxide, opening up a route for uncoupled cycling.

4. Conclusions

Insertion of the simplest possible scheme for a substrate-induced conformational change into the E_1E_2 model gives rise to the expanded scheme in Fig. 5, a network of coupled and uncoupled translocation paths containing no intermediate not implicit in the original model. Only conformational changes responsible for an ordered reaction (as well as carrier reorientation) are shown in the scheme. Details of individual reactions are omitted (see Refs. [1–4,25]). Thus S, representing the transported substrate, is actually two calcium ions, which on the outside add to the pump in a complicated cooperative process. ATP reacts with two forms of external complex, not a single form, and conformational changes accompany these reactions. A conformational change also occurs when internal calcium binds to the phosphorylated pump. Nevertheless, all the experimental observations can be placed within the context of the scheme. Another model, with calcium sites simultaneously exposed on both sides of the membrane, cannot be ruled out, though a complex containing 4 Ca^{2+} has not been observed.

While the substrates add in fixed order to the outward-facing carrier, addition to the inward-facing carrier is only partially ordered: that is, Ca^{2+} has only about 6-times greater affinity for the phosphorylated than for the free carrier, the half-saturation constants being about 1 mM and 6 mM, respectively [5]. Addition of Ca^{2+} to the inward-facing free carrier results in passive exit (Fig. 6B). The physiological concentration inside, estimated to be 2–5 mM [26], would set up a passive leak, were it not for the fact that in the presence of ATP, internal Ca^{2+} adds preferentially to the phosphorylated enzyme intermediate formed in the course of transport, inhibiting dephosphorylation [25] and, consequently, allowing Ca^{2+} to exit only through the coupled path involving ADP (Fig. 5A).

The expanded E_1E_2 model raises new issues. (i) The ratio of coupled to uncoupled flux, which gives a minimum for the increased binding force in a tight complex

formed during the transport cycle, is a key experimental parameter. (ii) Site-directed mutagenesis could, by shifting a conformational equilibrium, transform an active into a passive system, and such mutations pertain directly to the coupling mechanism (Fig. 6). (iii) In an asymmetrical ordered mechanism (Fig. 3) a binary complex (such as the phosphorylated carrier) may fail to move across the membrane simply because of the instability of that complex on the opposite side, but conformational immobility of the binary complex and vectorial catalysis in the ternary complex (as in a symmetrical ordered mechanism, Fig. 1) could be added, increasing the tightness of coupling. (iv) Substrates could both shift a conformational equilibrium and accelerate the rate at which the conformational change occurs (the former through preferential binding to one conformation, the latter through strong binding in the transition state).

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