Eur Biophys J (1998) 27: 255–262

ARTICLE

A. Reginald Waldeck · Karel van Dam · Jan Berden
Philip W. Kuchel

A non-equilibrium thermodynamics model of reconstituted Ca^{2+}-ATPase

Received: 19 June 1997 / Accepted: 3 December 1997

Abstract A non-equilibrium thermodynamics (NET) model describing the action of completely coupled or 'slipping' reconstituted Ca^{2+}-ATPase is presented. Variation of the coupling stoichiometries with the magnitude of the electrochemical gradients, as the ATPase hydrolyzes ATP, is an indication of molecular slip. However, the Ca^{2+} and H^+ membrane-leak conductances may also be a function of their respective gradients. Such non-ohmic leak typically yields 'flow-force' relationships that are similar to those that are obtained when the pump slips; hence, caution needs to be exercised when interpreting data of Ca^{2+}-ATPase-mediated fluxes that display a non-linear dependence on the electrochemical proton ($\Delta\tilde{\mu}_H$) and/or calcium gradients ($\Delta\tilde{\mu}_{Ca}$). To address this issue, three experimentally verifiable relationships differentiating between membrane leak and enzymic slip were derived. First, by measuring $\Delta\tilde{\mu}_H$ as a function of the rate of ATP hydrolysis by the enzyme. Second, by measuring the overall 'efficiency' of the pump as a function of $\Delta\tilde{\mu}_H$. Third, by measuring the proton ejection rate by the pump as a function of its ATP hydrolysis rate.

Key words Coupling stoichiometry · Electrogenicity · Slip · Calcium pumping · Proton ejection

Abbreviations *FCCP* Carbonylcyanide-*p*-trifluoromethoxy-hydrazone · *NET* Non-equilibrium thermodynamics · *PM* Plasma membrane · *SR* Sarcoplasmic reticulum

A. R. Waldeck [1] · P. W. Kuchel (✉)
Department of Biochemistry, The University of Sydney,
Sydney 2006, N.S.W., Australia
(e-mail: P.Kuchel@biochem.usyd.edu.au)

K. van Dam · J. Berden
E.C. Slater Institute for Biochemical Research,
The University of Amsterdam,
Plantage Muidergracht 12, 1081 TV Amsterdam,
The Netherlands

Present address:
[1] Biochemistry Unit, The Heart Research Institute,
Missenden Road 145–147, Camperdown 2050, N.S.W., Australia

Introduction

Ca^{2+}-ATPases exist in the plasma membranes of most cells and in the sarcoplasmic reticulum of myocytes where they pump Ca^{2+}, against a steep concentration gradient, out of the cytosol and into the lumen, respectively, while simultaneously counterporting H^+ ions. They share the catalytic properties of the 'P-type' family of ATPases but have distinctive regulatory properties. Both enzymes have a nearly absolute requirement for Mg^{2+} on the side from which Ca^{2+} is pumped, and are stimulated by K^+ and Na^+. The PM pump is stimulated by calmodulin, and a number of protein kinases, whereas the SR pump is not. [For reviews of the PM pumps see Roufogalis and Villalobo (1989), Schatzmann (1989), Carafoli (1994); and Schatzmann (1989), Inesi et al. (1990) and Jencks (1992), on the SR pumps.]

It is now generally established that the Ca^{2+}/ATP stoichiometries of the PM and SR enzymes are 1, and 2, respectively, reflecting their number of Ca^{2+} binding sites (Schatzmann 1989; Inesi et al. 1990; Carafoli 1994). Moreover, the reconstituted PM (Hao et al 1994) and SR (Yu et al. 1993) pumps are capable of establishing a membrane potential ($\Delta\psi$), while operating with $H^+/Ca^{2+}=1$.

A point of contention surrounding the experimental determination and theoretical foundations of coupling stoichiometries, is that of 'molecular slip' or 'intrinsic uncoupling' (Pietrobon and Caplan 1989). It has been suggested to occur in Ca^{2+}-ATPases by virtue of the coupling stoichiometries varying with the magnitude of the transmembrane electrochemical gradients (Roufogalis and Villalobo 1989; Hao et al. 1994). In a system coupled on the atomic or molecular level, integral values for the coupling stoichiometries are expected; thus, measuring non-integer values is a first indication of a slipping pump. To address this matter in more detail we devised a NET model of a reconstituted Ca^{2+}-ATPase that is subject to molecular slip; for reviews see Katchalsky and Curran (1974); Rottenberg (1979); Caplan and Essig (1983); Westerhoff and van Dam (1987); Pietrobon and Caplan (1989). Using the model, we

estimated the extent of slip in the PM Ca^{2+}-ATPase from steady state H^+ flow measurements (accompanying paper in this issue). In addition, mathematical expressions that may be used in experimental-data analysis to distinguish between slip and leak processes in Ca^{2+}-ATPase-liposomes were derived; protocols for their experimental verification are also presented.

Methods

Algebraic manipulations used in the derivation of expressions were carried out using the functions Eliminate, Collect, and Simplify in *Mathematica* (Wolfram 1991).

Model

Model without slip

The NET-based model of the reconstituted PM and SR membrane Ca^{2+}-ATPases is developed by considering the rate of entropy production (σ; units J K^{-1} s^{-1}), or rate of dissipation of free energy (Φ; units J s^{-1}) (Katchalsky and Curran 1974), in an 'idealised' Ca^{2+}-ATPase-liposome:

$$\Phi = T\,\sigma = J_P\,A_P - J_{Ca}\,\Delta\tilde{\mu}_{Ca} - J_H\,\Delta\tilde{\mu}_H \qquad (1)$$

where T denotes the absolute temperature (K), and A_P (=$-\Delta G_P$), $\Delta\tilde{\mu}_{Ca}$, and $\Delta\tilde{\mu}_H$ denote reaction affinity (negative Gibbs free energy change) associated with the ATP hydrolyzing activity of the enzyme, and the electrochemical Ca^{2+} and H^+ gradients, respectively (all units J mol^{-1}). The flux J_P (units mol s^{-1}) is the rate of 'scalar' flow (reaction rate) of ATP hydrolysis, and J_{Ca} and J_H are the respective *total* transmembrane flow rates (fluxes through space, normalized with respect to the membrane area) of Ca^{2+} and H^+. The term "total" refers to the sum of ATPase-mediated and leak fluxes (*vide infra* and Fig. 1). Note that ATP hydrolysis is a highly exergonic ('downhill') reaction, so the sign of ΔG_P is negative. The 'uphill' processes of inward Ca^{2+} and outward H^+ translocation bear a negative sign (see Fig. 1 for details on the sign conventions). This does not violate the second law of thermodynamics, $\Phi \geq 0$, as long as the sum of the products of flows and conjugate thermodynamic forces is greater than zero (Katchalsky and Curran 1974). The assumptions underlying Eq. (1), pertaining to the 'idealization' of the thermodynamic system (proteoliposome), have been discussed by Westerhoff et al. (1979).

Now consider a set of linear phenomenological rate-equations describing the completely coupled ATPase-mediated Ca^{2+} pumping and H^+ ejection (noting the sign conventions):

$$J_P = L_P\,A_P - L_{PCa}\,\Delta\tilde{\mu}_{Ca} - L_{PH}\,\Delta\tilde{\mu}_H \qquad (2a)$$

$$J_P^{Ca} = L_{CaP}\,A_P - L_{CaCa}\,\Delta\tilde{\mu}_{Ca} - L_{CaH}\,\Delta\tilde{\mu}_H \qquad (2b)$$

$$J_P^{H} = L_{HP}\,A_P - L_{HCa}\,\Delta\tilde{\mu}_{Ca} - L_{HH}\,\Delta\tilde{\mu}_H \qquad (2c)$$

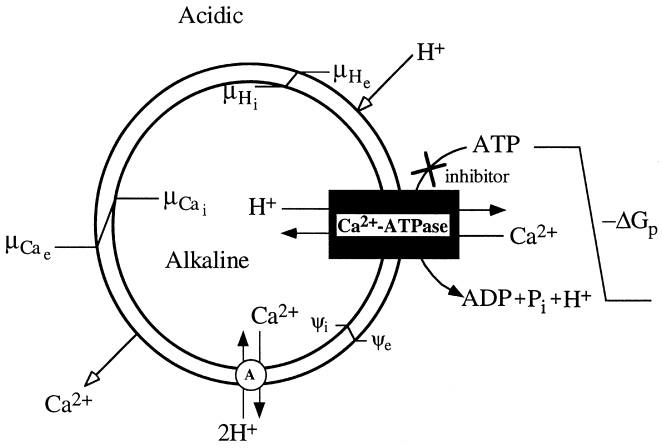

Fig. 1 Schematic representation of a Ca^{2+}-ATPase liposome. The orientation of the protein is such that externally added ATP initiates enzyme catalysis. The pump is a 'black box' in a membrane which has some intrinsic Ca^{2+} and H^+ conductance. Transmembrane H^+ and Ca^{2+} leaks are represented by arrows with open heads. Ca^{2+} pumping and H^+ ejection are represented by *arrows* with closed heads. Inwardly and outwardly directed transmembrane fluxes are taken to be negative and positive, respectively; the scalar flow of ATP hydrolysis is taken to be positive, as its 'conjugate' force, A_P (=$-\Delta G_P$), drives the 'uphill' transport of Ca^{2+}, and H^+ (see "Model without slip"). The chemical (μ) gradients (Δ) and their direction corresponding to Ca^{2+}, H^+, and ATP, and the electrical (ψ) gradient are represented by the symbol ⌒⌣. These forces are defined as follows: $\Delta\mu_{Ca} = \mu_{Ca_i} - \mu_{Ca_e}$; $\Delta\mu_H = \mu_{H_i} - \mu_{H_e}$; and $\Delta\psi = \psi_i - \psi_e$ (subscript i and e denote internal and external, respectively). Thus, we have for $\Delta\tilde{\mu}_X$ (X = H^+, Ca^{2+}): $\Delta\tilde{\mu}_X = R\,T\,\ln([X_i]/[X_e]) + Z\,F\,\Delta\psi$, which for H^+ corresponds to: $\Delta\tilde{\mu}_H = F\,\Delta\psi - 2.3\,R\,T\,\Delta pH$. $\Delta G_P = \Delta G_P'^0 - RT\ln\{[ATP]/([ADP][P_i]([H^+]/10^{-pH}))\}$, where $\Delta G_P'^0$ denotes the standard Gibbs free energy for the hydrolysis of ATP at pH 7.0 (–30.5 kJ mol^{-1}; Eisenberg and Crothers 1979), and R, T, and Z denote the universal gas constant, absolute temperature, and number of coulombic charge, respectively. Thus, the system is defined so that all three forces involved, namely the Ca^{2+}-gradient, the H^+-gradient, and the transmembrane voltage, are counteracting the ATP hydrolysing activity of the enzyme (i.e., they induce a 'backpressure' effect): $\Delta\mu_{Ca} > 0$, $\Delta\mu_H < 0$ (i.e., $\Delta pH > 0$), and $\Delta\psi > 0$. The action of the ionophore A23187 and the inhibition of ATP hydrolysis by orthovanadate (PM pump) or thapsigargin (SR pump), are indicated by the symbols Ⓐ and ×, respectively

J_P^{Ca}, and J_P^{H} denote the ATPase-induced (subscript P) flows of Ca^{2+} and H^+ (superscripts Ca, H), respectively. L_{II} and L_{IJ} (L_{JI}; mol^2 s^{-1} kJ^{-1}) are the so-called 'straight' and 'cross' coefficients (Katchalsky and Curran 1974), respectively.

When 'Onsager reciprocity' is assumed to apply to Eqs. (2a–c), $L_{IJ} = L_{JI}$ for all species of I and J (Katchalsky and Curran 1974). However, reciprocal (proportional) flow-force relationships are strictly valid for the near-equilibrium domain only; i.e., when $|\Delta G| \ll RT$. Experimentally this restriction translates into $\Delta G \leq \sim \pm 1.5$ kJ mol^{-1}, which would place most systems of biological interest, including Ca^{2+}-ATPases, outside the applicability of the theory. However, upon closer inspection the linear approximation is shown to hold rather well, even far from equilibrium (*vide infra*).

The domain of linearity

By imposing boundary conditions, such as conservation of mass, flow-force relationships of enzyme-catalysed and chemical reactions can be shown to be described by a simple hyperbolic-tangent function (ratio of double exponentials; for reviews see Rottenberg 1979; Westerhoff and van Dam 1987; Pietrobon and Caplan 1989; Walz 1990). Thus, a plot of J as a function of ΔG (A) has three regions: at very high positive and negative values of ΔG, the reaction rate is almost independent of ΔG, and in between these parts of the curve there is a region where the rate changes smoothly from its lowest to its highest value. Thus, there exists a quasi-linear region around the inflection point. The linearity extends over an ~7 kJ mol^{-1} neighbourhood of the equilibrium-value of ΔG. The corresponding error in the reaction rate (flux; J) is < 15% for 75% of the range of possible rates (Westerhoff and van Dam 1987).

NET parameters

The phenomenological coefficients in Eq. (2a–c) determine the phenomenological stoichiometry, Z, the 'degree of coupling', q, and the efficiency of energy conversion, η, of the coupled processes (Katchalsky and Curran 1974; Caplan and Essig 1983). We consider the 'static head' with respect to Ca^{2+} pumping in which the power (J s^{-1}) term $J_P^{Ca}\,\Delta\tilde{\mu}_{Ca}=0$. Static head refers to the condition $J_P^{Ca}=0$; i.e., a vanishing Ca^{2+} pumping rate. The other situation for which $J_P^{Ca}\,\Delta\tilde{\mu}_{Ca}=0$, is called 'level flow'; for this condition $\Delta\tilde{\mu}_{Ca}=0$. Near equilibrium Z and q, under static head conditions (sh), are defined by:

$$Z_{sh} = Z_{HP} = \sqrt{L_{HH}/L_P} \tag{3}$$

$$q_{sh} = q_{HP} = L_{PH}/\sqrt{L_P\,L_{HH}} \tag{4}$$

From Eqs (2a–c), (3), and (4) we obtain an expression for the overall efficiency of energy conversion under the static head condition:

$$\begin{aligned}\eta_{sh} = \eta_{HP} &= -(J_P^H\,\Delta\tilde{\mu}_H)/(J_P\,A_P) = j\,\chi \\ &= -Z\,\chi\,(Z\,\chi+q)/(q\,Z\,\chi+1)\end{aligned} \tag{5}$$

where j and χ denote flow and thermodynamic-force ratio, respectively. The optimal conditions for biological free energy transduction are not necessarily the state(s) of maximal efficiency (Stucki 1980a, b; Juretiç and Westerhoff 1987). In fact, for Ca^{2+}-ATPases, the maintenance of a transmembrane Ca^{2+} gradient is physiologically very important, so the pumps are likely to operate in a situation that is close to a static head one, and thus appear to be operating with almost zero efficiency.

Incorporating biochemical knowledge and far from equilibrium considerations

We continue our treatment by incorporating biochemical knowledge of Ca^{2+}-ATPases into the NET model and addressing the issue of remoteness from equilibrium. First, we consider the coupling stoichiometries. During a turnover cycle of the pump n_P^{Ca} calcium ions and n_P^H protons are transported across the membrane; thus, n_P^{Ca} and n_P^H denote the Ca^{2+}/ATP and H^+/ATP coupling stoichiometries. The "convention" taken here is that superscript represents numerator, and subscript represents denominator; e.g., n_P^{Ca} stands for the number of Ca^{2+} ions per (over) ATP molecule. Thus, the H^+/Ca^{2+} stoichiometry is n_{Ca}^H, and is given by n_P^H/n_P^{Ca}. As a result, the bioenergetics of PM and SR Ca^{2+}-ATPases is characterised by two independent coupling stoichiometries. Second, we make use of the experimental observation that both $\Delta\tilde{\mu}_{Ca}$ and $\Delta\tilde{\mu}_H$ inhibit J_P; i.e., both thermodynamic forces exert a 'backpressure' effect on the rate of ATP hydrolysis for the PM (Villalobo and Roufogalis 1986; Wang et al. 1989; Hao et al. 1994) and SR pumps (Yu et al. 1993; Yu et al. 1994; Yu and Inesi 1995). Therefore, the total thermodynamic force is taken to be $A_P - n_P^{Ca}\,\Delta\tilde{\mu}_{Ca} - n_P^H\,\Delta\tilde{\mu}_H$, where n_P^{Ca} and $\Delta\tilde{\mu}_H$ carry a negative sign (see legend of Fig. 1). Third, by assuming Onsager reciprocity to hold, we may write $L_{PCa}=L_{CaP}=n_P^{Ca}\,L_P$ and $L_{PH}=L_{HP}=n_P^H\,L_P$.

Next, we introduce concepts allowing far-from-equilibrium reactions to be characterised by *apparent* proportional flow-force relationships which, in turn, account for the known saturation of enzymic reactions (Westerhoff and van Dam 1987; Pietrobon and Caplan 1989). The point (region) in the J-versus-ΔG curve where saturation sets in (see section "The domain of linearity") is treated by introducing the quantity $\Delta G^{\#}$ ($-A^{\#}$), where $\Delta G_P^{\#}=(\Delta G_P)_{J_P=0}$. Thus, $\Delta G_P^{\#}$ is a measure of the off-set from equilibrium in kJ mol^{-1} of the $J_P-\Delta G_P$ relationship. We proceed by introducing the quantity $\Delta G_P^{£}$ ($=\Delta G_P-\Delta G_P^{\#}$), which may be viewed as the effective driving force ($\Delta G_P^{£}=-A_P^{£}$) driving the uphill translocations of Ca^{2+} and H^+. As a result, the value of the "activity" coefficient, L_P, changes; and because this change may not affect $\Delta\tilde{\mu}_{Ca}$ and $\Delta\tilde{\mu}_H$ equally, it is necessary to introduce 'asymmetry' coefficients, γ_P^{Ca} and γ_P^H [see Eqs. (7a) and (7b)]. Thus, the equations describing the 'strict mechanistically' coupled action of the pump near, and far from equilibrium, are obtained by using Eqs. (2a–c) and the above considerations, to give:

$$J_P = L_P\,(A_P^{£} - n_P^{Ca}\,\gamma_P^{Ca}\,\Delta\tilde{\mu}_{Ca} - n_P^H\,\gamma_P^H\,\Delta\tilde{\mu}_H) \tag{6a}$$

$$J_P^{Ca} = n_P^{Ca}\,L_P\,(A_P^{£} - n_P^{Ca}\,\gamma_{Ca}^P\,\Delta\tilde{\mu}_{Ca} - n_P^H\,\gamma_P^H\,\Delta\tilde{\mu}_H) \tag{6b}$$

$$J_P^H = n_P^H\,L_P\,(A_P^{£} - n_P^{Ca}\,\gamma_P^{Ca}\,\Delta\tilde{\mu}_{Ca} - n_P^H\,\gamma_H^P\,\Delta\tilde{\mu}_H) \tag{6c}$$

where

$$\gamma_P^{Ca} = \frac{\delta(\Delta G_P)_{\Delta\tilde{\mu}_{Ca}}}{\delta(\Delta\tilde{\mu}_{Ca})_{\Delta G_P}} \tag{7a}$$

and

$$\gamma_P^H = \frac{\delta(\Delta G_P)_{\Delta\tilde{\mu}_H}}{\delta(\Delta\tilde{\mu}_H)_{\Delta G_P}} \quad (7b)$$

Note that $\gamma_X^Y = 1/\gamma_Y^X$ in Eqs. (6a–c). In an 'ideal' situation, J_P will change in proportion to a change in A_P (and $\Delta\tilde{\mu}_{Ca}$ and $\Delta\tilde{\mu}_H$). According to these criteria the SR pump is 'ideal'; i.e., the maximum rates of ATP hydrolysis and synthesis are nearly identical, making it a 'readily kinetically-reversible' enzyme. On the other hand, the rate of ATP synthesis by the PM pump is only 0.5–1% of the forward rate, rendering it a 'kinetically irreversible' enzyme (Schatzmann 1989 and references therein) and thus 'non-ideal'. In the following, we assume, for the sake of simplicity, that $\gamma_P^{Ca} = \gamma_P^H = 1$. Thus, the theory applies strictly only to the SR enzyme, but this simplification does not invalidate the conclusions made regarding the distinction between molecular slip and membrane leaks in PM Ca^{2+}-ATPase liposomes.

Introducing molecular slip

ATPase slip, and the membrane leaks of Ca^{2+} and H^+ are incorporated into the definitions of the rate coefficients L_P^s, L_{Ca}^l, and L_H^l. Thus the matrix-form of the reaction-flux equations is:

$$\begin{pmatrix} J_P \\ J_{Ca} \\ J_H \end{pmatrix} = \quad (8)$$

$$\begin{pmatrix} L_P + L_P^s & -n_P^{Ca} L_P & -n_P^H L_P \\ n_P^{Ca} L_P & -\left[(n_P^{Ca})^2 L_P + L_{Ca}^1\right] & -n_P^{Ca} n_P^H L_P \\ n_P^H L_P & -n_P^H n_P^{Ca} L_P & -\left[(n_P^H)^2 L_P + L_H^1\right] \end{pmatrix} \begin{pmatrix} A_P \\ \Delta\tilde{\mu}_{Ca} \\ \Delta\tilde{\mu}_H \end{pmatrix}$$

where $J_{Ca} = J_P^{Ca} + J_{Ca}^1$ and $J_H = J_P^H + J_H^1$; J_{Ca}^1 and J_H^1 denote leak currents (flows) of Ca^{2+} and H^+, respectively. L_P^s is the slip-rate coefficient, whereas L_P is the strict mechanistically coupled ATPase rate coefficient. Note that L_P^s does not take into account the *kinetic* properties of molecular slip (Hill 1977); e.g., see those of the SR pump (Pickart and Jencks 1983; Tanford 1984; Walz and Caplan 1988; Krupka 1994; Yu and Inesi 1995); see also the Discussion.

Equation (8) constitutes a minimal model of the ATPase, in the sense that leaks of 'bulk' ions, such as K^+ and Cl^-, etc. are not taken into account (see c.f. Westerhoff et al. 1979). Nevertheless, as shown below, the present model contains a sufficient number of features to yield experimentally verifiable relationships that will allow the distinction of ATPase slip from membrane leak. In the following we illustrate the experimental application of Eq. (8) by addressing the issues of non-zero membrane leak and ATPase slip.

Experimental determination of the coupling stoichiometries at steady state

We estimate n_P^H in the *absence* of the ionophore A23187 from steady state NMR data in its presence (accompanying paper in this issue), as follows:

$$n_P^H = J_P^H / J_P = (J_H^e - J_H^s + J_H^1)/J_P \quad (9)$$

where the subscript e denotes, external; expressions for J_H^e and J_H^s are given in Eqs. (7b) and (7c) in the accompanying paper in this issue. Note that in these experiments, extravesicular H^+ production has been measured, and hence we need to correct for the scalar proton production *in* the external medium (i.e., substract it), and H^+ leak *from* the external medium (i.e., add it on). We estimate J_H^1 from an independent measurement of L_H^1 (Kamp 1989) and $\Delta\tilde{\mu}_H^{ss}$ (superscript ss denotes steady state), as follows:

$$J_H^1 = L_H^1 \Delta\tilde{\mu}_H^{ss} = L_H^1 (F \Delta\psi^{ss} - R\,T\,\Delta pH^{ss}) \quad (10)$$

where F denotes Faraday's constant. An estimate of n_P^H in the presence of A23187 has been made in the accompanying paper in this issue. An estimate of $\Delta\tilde{\mu}_H^{ss}$ in the absence of A23187 may be obtained from estimates of $\Delta\psi$ and ΔpH[1], H^+ flow measurements in the absence of the ionophore, and assuming proportional flow-force relationships. By assuming the latter, a change of J_P is assumed to be proportional to the same change of $\Delta\tilde{\mu}_H^{ss}$ (and J_H^p). Therefore, $\Delta\tilde{\mu}_H^{ss}$ in the absence of A23187 is the product of the uncoupler stimulation factor, or control ratio of the enzyme; J_P (+A23187)/J_P (–A23187) = 4 (accompanying paper in this issue) and $\Delta\tilde{\mu}_H^{ss}$ in the presence of A23187. The quantities used in Eqs. (9) and (10) are given in Table 1, and they yield $n_P^H = 1.9 \pm 0.3$.

Estimating the extent of molecular slip

Using Eq. (8) we can estimate the maximal extent of slip (L_p^s/L_p) in the PM Ca^{2+}-ATPase, by taking the system in the absence of A23187 (–) to be close to static head, and the system in the presence of the ionophore (+) to be approximated by level flow. Thus, from the equation for J_P we obtain for the control ratio of the enzyme:

$$(J_P^+/J_P^-) = (A_P^+/A_P^-)\,(1 + (L_p^s/L_p)) \quad (11)$$

A_P^- and A_P^+ estimated from data (in the accompanying paper in this issue) amounted to ~61 kJ mol^{-1} and ~52 kJ mol^{-1}, respectively; thus $L_p^s/L_p \leq 0.4$. In other words, for every five coupled turnovers leading to Ca^{2+} and H^+ translocation (i.e., work) there may be as many as two uncoupled ones. In the latter, the energy associated with the hydrolysis of ATP would be lost as heat (i.e., dissipated).

Slip versus leak

In the following we derive equations that allow molecular slip, intrinsic to the ATPase, to be distinguished from,

Table 1 Quantities used for estimating n_P^H at steady state in the absence of A23187[a]

Quantity	Value	Units
J_P (–A23187)	133±21[a]	nmol (mg protein)$^{-1}$ min
J_H^e (–A23187)	46±7[a]	nmol (mg protein)$^{-1}$ min
Control ratio	4[a]	–
$n_H^{P,scalar}$	0.8[b]	–
L_H^1	100[c]; 2.25	nmol (mg PL)$^{-1}$ (pH)$^{-1}$ s^{-1}; µmol (mg protein)$^{-1}$ (pH)$^{-1}$ min^{-1}
J_H^1	315[d]	nmol (mg protein)$^{-1}$ min
ΔpH	–0.03[e]	pH
$\Delta\psi$	37; 0.003[e]	µV; pH
$\Delta\tilde{\mu}_H^{ss}$ (–A23187)	0.14; 0.36[d]	pH; kJ mol^{-1}
$\Delta\tilde{\mu}_H^{ss}$ (+A23187)	0.035; 0.090[d]	pH; kJ mol^{-1}
Sample volume	2[a]	ml
Protein concentration	40±6[a]	mg ml^{-1}
Lipid concentration	15[a]	mg ml^{-1}

[a] From (unpublished results).
Control ratio = J_P (–A23187)/J_P (+A23187)
[b] From Nishimura et al. (1962)
[c] From Kamp (1989)
[d] Estimated in this work
[e] See Footnote [1]

membrane leak, intrinsic to the membrane-proper. Thus, slip and leak occur in parallel, and we make use of this property of the system to distinguish between the two processes. Three possible theoretical procedures involving the $J_P - \Delta\tilde{\mu}_H$ flow-force relationship, the overall thermodynamic efficiency (η_{HP}) of the pump, and the relationship between J_H and J_P, and their respective experimental protocols are discussed.

Flow-force relationships

We cannot obtain any further insight into the issue of distinguishing molecular slip from the membrane leaks using Eq. (8) per se. Therefore, to overcome this problem we assume that molecular slip in the ATPase is dependent on *which ever* thermodynamic force ($\Delta\tilde{\mu}_{Ca}$ or $\Delta\tilde{\mu}_H$) generates the backpressure, in the absence of the other. Also, the rate of leakage of the coupling ions will be dependent on the *magnitude* of the particular thermodynamic force (Pietrobon et al. 1981; Jackson 1982) operating in the system (again, in the absence of the other), but not on the choice of the force. The theoretical procedure entails rearranging Eq. (8) for J_P, which yields:

$$J_P = (L_P((1+(L_P^s/L_P))\, A_P - n_P^{Ca}\, \Delta\tilde{\mu}_{Ca} - n_P^H\, \Delta\tilde{\mu}_H) \qquad (12)$$

Applying the static head condition, $J_H = 0$, to Eq. (8) we obtain for L_P:

$$L_P = L_H^1\, \Delta\tilde{\mu}_H / (n_P^H (A_P - n_P^{Ca}\, \Delta\tilde{\mu}_{Ca} - n_P^H\, \Delta\tilde{\mu}_H)) \qquad (13)$$

Inserting Eq. (13) into Eq. (12) and keeping the factor (L_P^s/L_P) separate, yields:

$$J_P = \frac{L_H^1\, \Delta\tilde{\mu}_H}{n_P^H} \left(1 + \frac{(L_P^s / L_P)}{1 - (n_P^{Ca}\, \Delta\tilde{\mu}_{Ca} + n_P^H\, \Delta\tilde{\mu}_H)/A_P}\right) \qquad (14)$$

As expected, Eq. (14) is identical to the one obtained for an H^+-ATPase in a mitochondrial membrane (Westerhoff and van Dam 1987) except for the term corresponding to Ca^{2+} transport ($n_P^{Ca}\, \Delta\tilde{\mu}_{Ca}/A_P$). An identical equation is obtained when the steady state condition, $J_{Ca} = 0$, is used, except that the term in front of the brackets reads ($L_{Ca}^1\, \Delta\tilde{\mu}_{Ca}/n_{Ca}^P$). Figure 2 shows the variation of $\Delta\tilde{\mu}_H$ with J_P according to Eq. (14), for $\Delta\tilde{\mu}_{Ca} = 0$.

The experimental procedure would encompass measuring the dependence of the steady state $\Delta\tilde{\mu}_{Ca}$ and $\Delta\tilde{\mu}_H$ on J_P, in the absence of the other force ($\Delta\tilde{\mu}_H$ and $\Delta\tilde{\mu}_{Ca}$, respectively). J_P may be titrated by using an inhibitor of the Ca^{2+}-ATPase; e.g., orthovanadate and thapsigargin for the PM and SR pumps, respectively. The different shapes of the curves, would not only demonstrate slip in the ATPase, but would also indicate which force is more efficient in inducing it; i.e., the parameter L_P^s/L_P may be greater for $\Delta\tilde{\mu}_{Ca}$ than for $\Delta\tilde{\mu}_H$, or vice versa (see Fig. 2).

The thermodynamic efficiency

In the following we present a theoretical procedure that enables molecular slip to be distinguished from membrane leaks by measuring η_{HP} (Eq. (5)) as a function of $\Delta\tilde{\mu}_H$. No further assumptions need to be made. Applying the static head condition, $J_{Ca} = 0$ Eqs. (2a–c) and (initially) writing the forces as a function of the flows (Rottenberg 1979), and applying Onsager reciprocity, we obtain:

$$J_P = L_P\, \Delta G_P - L_{PH}\, \Delta\tilde{\mu}_H \qquad (15a)$$

$$J_H = L_{PH}\, \Delta G_P - L_{HH}\, \Delta\tilde{\mu}_H \qquad (15b)$$

The phenomenological coefficients of the two-flow-force system (Eqs. (15a–b)) are then expressed in terms of the coefficients in Eq. (8) by applying the same static head

[1] We estimated an excess 0.6 H^+ in the presence of A23187, to be equivalent to ~277 nM H^+, given an average radius of ~95 nm for the Ca^{2+}-ATPase SUV. The depletion of 0.6 protons from the extravesicular medium would not appreciably change the pH in that compartment, as $V_e \gg V_i$; and thus, the ΔpH may be calculated to be 0.75 in the absence of buffering species. In the presence of 10 mM Hepes buffer at pH ~7.3, however, any ΔpH would be minimal. We may assume that $\beta_i \approx \beta_e \approx 10$ µmol H^+/pH unit (see accompanying paper in this issue), and hence, an excess of 277 nM H^+ would have resulted in a ΔpH of –0.03 (–0.0277) U (acidic inside). Using a Ca^{2+}/ATP stoichiometry of 0.83 (Wang et al. 1989), we estimated an intraliposomal depletion of 0.17 Ca^{2+} in the presence of A23187. The magnitude of the membrane potential ($\Delta\psi$) was calculated from the equation $Q = C\, \Delta\psi$. Thus, $\Delta\psi = ([H^+] - 2\,[Ca^+]) \times ((R_{SUV} \times F)/(3 \times C))$, where C denotes membrane capacitance per unit area (~1 µF cm^{-2} for lipid bilayers), Q, F, and R denote the amount of charge in coulombs, Faraday's constant, and radius, respectively. Its magnitude would be of the order of 0.01–0.1 (0.037) mV (positive inside)

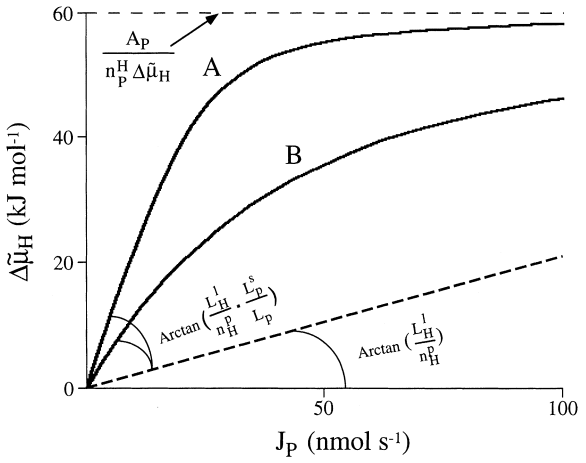

Fig. 2 Graphs of the electrochemical proton gradient, $\Delta\tilde{\mu}_H$, versus the rate of ATP hydrolysis of the Ca^{2+}-ATPase, J_P, according to Eq. (15). The values of the parameters are: A_P = 60 kJ mol^{-1}; $n_P^{Ca} = n_P^H = 1$; $L_H^1 = 5\times10^{-10}$ mol^2 s^{-1} kJ^{-1} (from Table 2); $\Delta\tilde{\mu}_{Ca}$ = 0 kJ mol^{-1}. *A* $L_p^s/L_p = 0.1$; i.e., the "activity" of the slip cycle is ten-fold less than the corresponding coupled cycle. *B* $L_p^s/L_p = 1.0$. Thus, the parameter L_p^s/L_p determines the shape of the hyperbola, and its magnitude is expected to be different when $\Delta\tilde{\mu}_{Ca}$ is the force exerting the back-pressure (see also text). The *dashed line* depicts the situation in the absence of slip (i.e., $L_P^s = 0$); thus, the flow-force relationship is linear. Note that (1) the extent of slip L_P^s/L_P is given by the ratio of the actual slope to the slope that is expected from the leak coefficient and the coupling stoichiometry, and (2) in the case of a slipping pump, the deviation from linearity in the flow-force relationship becomes more evident as $n_P^H \Delta\tilde{\mu}_H$ approaches A_p

condition to that equation, and eliminating $\Delta\tilde{\mu}_{Ca}$ in the expressions obtained for J_P and J_H. The analysis reveals that ATPase slip, and leak of Ca^{2+} and H^+ have different effects on L_P, L_{HH}, and L_{PH}:

$$L_P = L_P^s + L_t \tag{16a}$$

$$L_{PH} = n_P^H L_t \tag{16b}$$

$$L_{HH} = L_H^1 + (n_P^H)^2 L_t \tag{16c}$$

$$L_t = L_P \left(1 - \frac{1}{1 + (L_{Ca}^1 / ((n_P^{Ca})^2 L_P))}\right) \tag{16d}$$

The predicted qualitative effects of ATPase slip, Ca^{2+} and H^+ leaks on the phenomenological coefficients, and the parameters Z, and $(\Delta\tilde{\mu}_H)_{sh}$ are given in Table 2 (subscript sh denotes static head). Note that Z provides information on which of the three processes contributes to q_{HP} being less than one (see Table 2).

Figure 3 shows the variation of η_{HP} as a function of $\Delta\tilde{\mu}_H$ for decreasing values of q_{HP}. The clearest difference between the curves is that $\Delta\tilde{\mu}_H$ at static head, i.e., when $J_H = \eta_{HP} = 0$, changes when the Ca^{2+} and H^+ leaks through the membrane are varied, but not when slip in the ATPase is affected. Also, the force at which the η_{HP} is maximal for a given q_{HP}-value is different for ATPase slip, Ca^{2+} leak, and H^+ leak.

Table 2 Qualitative effects[a] of ATPase slip, and Ca^{2+} and H^+ leaks on the phenomenological coefficients, Z, and $(\Delta\tilde{\mu}_H)_{sh}$

Process that decreases q	Effect on				
	L_{PP}	L_{PH}	L_{HH}	Z	$(\Delta\tilde{\mu}_H)_{sh}$
ATPase slip	+	=	=	–	=
Ca^{2+} leak	–	–	–	–	–
H^+ leak	=	=	+	+	–

[a] The symbols +, –, and = denote a positive, negative, and no effect on the parameter of interest; the subscript sh denotes static head

From an experimental point of view, η_{HP} is to be measured as a function of $\Delta\tilde{\mu}_H$ at constant A_P. The latter may be clamped using ADP and glucose 6-phosphate in the presence of hexokinase; this system then functions as an ATP-regenerating system. The $\Delta\tilde{\mu}_H$ may be varied by modulating J_P with an inhibitor. The overall efficiency (η_{HP}) may be determined by measuring simultaneously J_P^H, J_P, $\Delta\tilde{\mu}_H$, and A_P by NMR (accompanying paper in this issue), or by optical methods (Karon et al. 1995). The predicted effects of Ca^{2+} and H^+ leak may be ascertained by adding limiting concentrations of CYCLEX-2E (and K^+-valinomycin) and a protonophore, respectively.

The relationship between J_H and J_P

ATPase slip and Ca^{2+} or H^+ leaks should also exert different effects on the relationship between J_H and J_P when $J_{Ca} = 0$. Eliminating $\Delta\tilde{\mu}_H$ in Eq. (15a), substituting the resulting equation into Eq. (15b), and substituting the phenomenological coefficients by Eqs. (16a–d), we obtain:

$$J_H = \alpha J_P - \beta A_P \tag{17}$$

where:

$$\alpha = n_P^H + \left(\frac{L_H^1}{n_P^H}\right)\left(\frac{1}{L_P} + \frac{(n_P^{Ca})^2}{L_{Ca}^1}\right) \tag{18a}$$

$$\beta = \frac{L_H^1}{n_P^H}\left\{1 + L_P^s\left(\frac{1}{L_P} + \frac{(n_P^{Ca})^2}{L_{Ca}^1} + \frac{(n_P^H)^2}{L_H^1}\right)\right\} \tag{18b}$$

When J_H is plotted as a function of J_P, α corresponds to the slope and β corresponds to the intercept with the abscissa, when A_P is clamped experimentally. In case of increasing leak (H^+ or Ca^{2+}), both the slope and the intercept are effected; this would cause lines determined with different uncoupler concentrations to intersect. However, when the pump is slipping the intercept with the abscissa would change but the slope would remain the same (α does not contain L_P^s). Conveniently, distinguishing leak from slip using Eqs. (17)–(18) does not require an estimate of $\Delta\tilde{\mu}_H$.

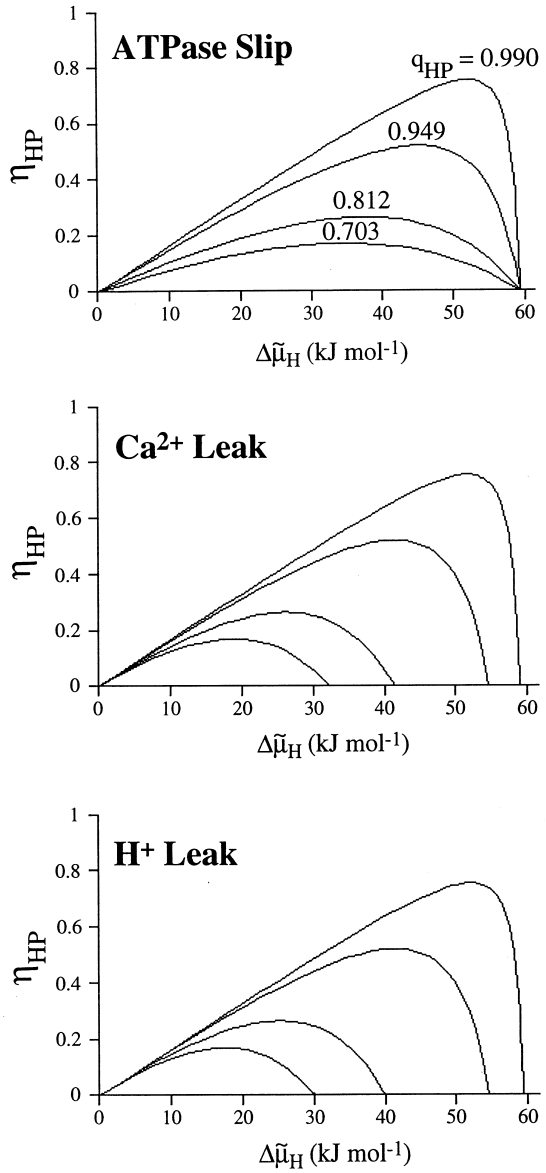

Fig. 3 The effect of ATPase slip (L_P^s), and Ca^{2+} (L_{Ca}^1) and H^+ leaks (L_H^1) on the overall thermodynamic efficiency of the Ca^{2+}-ATPase (η_{HP}) for various degrees of coupling (q_{HP}), as a function of $\Delta\tilde{\mu}_H$. The different q_{HP}-values (see figure) were obtained by varying one of L_P^s, L_{Ca}^1, or L_H^1, while keeping the other two parameters constant. The parameter values that were used in the individual simulations, and which gave rise to decreasing values of q_{HP}, were: Slip; L_P^s = 0.01, 0.1, 0.5, and 1, respectively; L_{Ca}^1 = 1; L_H^1 = 0.01. Ca^{2+} leak; L_{Ca}^1 = 5.5, 1.0, 0.2, and 0.1, respectively; L_P^s = 0.01; L_H^1 = 0.1. H^+ leak; L_H^1 = 0.01, 0.1, 0.5, and 1, respectively; L_P^s = 0.01; L_{Ca}^1 = 1 (all L's have units mol^2 s^{-1} kJ^{-1}). The values of the parameters that were kept constant throughout were: A_P = 60 kJ mol^{-1}; $n_H^P = n_{Ca}^P$ = 1; L_P = 1000 mol^2 s^{-1} kJ^{-1}. Arbitrary values were used for L_P^s, L_{Ca}^1, and L_H^1, because only the value of the latter is known (see legend of Fig. 2); however, this is of no consequence to the qualitative effects to be demonstrated

Discussion

In the present work we have presented a NET description of the vectorially oriented Ca^{2+}-ATPase that allows the distinction between the effects of molecular slip and ion leaks, on J_P or J_H. Notably, the derived linear force-flow relationships remain intuitively clear, and are readily scrutinized experimentally.

The effects of enzymic slip and membrane H^+ leak in respiring mitochondria have been modelled successfully when compared with experimental results (Pietrobon et al. 1983; Westerhoff and van Dam 1987; Groen et al. 1990). Therefore, a similar approach was used in the present work to derive expressions allowing Ca^{2+}-ATPase slip to be distinguished from membrane Ca^{2+} and H^+ leaks by measuring: (1) $\Delta\tilde{\mu}_H$ as a function of J_P [Eq. (14)]; (2) η_{HP} as a function of $\Delta\tilde{\mu}_H$ [Eqs. (5), (15–16)]; and (3) J_H as a function of J_P [Eqs. (17)–(18)].

As a comparison with the model presented in this work, we note that non-linear flow-force relationships in oxidative phosphorylation have been modelled by phenomena other than slipping pumps and/or non-ohmic H^+ leaks. The first such model entails the 'mosaic protonic coupling' hypothesis (Westerhoff et al. 1984) which considers the 'clustering' of respiratory enzymes; the second one relates to the heterogeneity in the degree of coupling between individual mitochondria in a mitochondrial preparation (Duszynski and Wojtczak 1985). Importantly, these phenomena are not envisaged to arise in proteoliposomes.

From a theoretical standpoint, the model may be used in a thermokinetic description (Pietrobon nd Caplan 1985; Walz and Caplan 1988; Walz 1990) of the (SR) ion pump to investigate (theoretically) the domain of linearity and proportionality of the flow-force relationships, as well as the 'kinetic (in)equivalence' of the input and output forces (see e.g., Pietrobon and Caplan 1985).

Acknowledgements This work was supported by a grant to P. W. K. from the Australian National Health and Medical Research Council. K. v. D. acknowledges the Foundation for Chemical Research (SON) which is subsidised by the Netherlands Organisation for Scientific Research (NWO). A. R. W. acknowledges the support of an Overseas Postgraduate Research Award/Scholarship.

References

Caplan SR, Essig A (1983) Bioenergetics and linear non-equilibrium thermodynamics: the Steady State. Harvard University Press, London

Carafoli E (1994) Biogenesis: plasma membrane calcium ATPase: 15 years of work on the purified enzyme. FASEB J 8:993–1001

Duszynski J, Wojtczak L (1985) The apparent non-linearity of the relationship between the rate of respiration and the protonmotive force of mitochondria can be explained by heterogeneity of mitochondrial preparations. FEBS Lett 182:243–248

Eisenberg D, Crothers D (1979) Physical chemistry with applications to the life sciences. Benjamin/Cummings, Menlo Park, California, p 382

Groen BH, Berden JA, van Dam K (1990) Differentiation between leaks and slips in oxidative phosphorylation. Biochim Biophys Acta 1019:121–127

Hao L, Rigaud J-L, Inesi G (1994) Ca^{2+}/H^{+} Countertransport and electrogenicity in proteoliposomes containing erythrocyte plasma membrane Ca-ATPase and exogenous lipids. J Biol Chem 269: 14268–14275

Hill T (1977) Free energy transduction in biology. Academic Press, New York

Inesi G, Sumbilla C, Kirtley ME (1990) Relationships of molecular structure and function in Ca^{2+}-transport ATPase. Physiol Rev 70: 749–760

Jackson JB (1982) Evidence that the ionic conductivity of the cytoplasmatic membrane of *Rhodopseudomonas capsulata* is dependent upon membrane potential. FEBS Lett 139: 139–143

Jencks WP (1992) On the mechanism of ATP-driven Ca^{2+} transport by the calcium ATPase of sarcoplasmic-reticulum. Ann NY Acad Sci 671: 49–57

Juretiç D, Westerhoff HV (1987) Variation of efficiency with free-energy dissipation in models of biological energy transduction. Biophys Chem 28: 21–34

Kamp F (1989) PhD Thesis. The University of Amsterdam, The Netherlands

Katchalsky A, Curran PF (1974) Nonequilibrium thermodynamics in biophysics, 2nd edn. Harvard University Press, Cambridge, Massachusetts

Krupka RM (1994) The application of vectorial coupling theory to the calcium pump. Biochim Biophys Acta 1193: 179–185

Nishimura M, Ito T, Chance B (1962) A sensitive and rapid method of determination of photophosphorylation. Biochim Biophys Acta 59: 177–182

Pickart CM, Jencks WP (1983) Energetics of calcium-transporting ATPase. J Biol Chem 259: 1629–1643

Pietrobon D, Azzone GF, Walz D (1981) Effect of funiculosin and antimycin A on the redox-driven H^{+}-pumps in mitochondria: on the nature of leaks. Eur J Biochem 117: 389–394

Pietrobon D, Zoratti M, Azzone GF (1983) Molecular slipping in redox and ATPase H^{+} pumps. Biochim Biophys Acta 723: 317–321

Pietrobon D, Caplan SR (1985) Flow-force relationships for a six-state proton pump model: intrinsic uncoupling, kinetic equivalence of input and output forces, and domain of approximate linearity. Biochemistry 24: 5764–5776

Pietrobon D, Caplan SR (1989) Use of non-equilibrium thermodynamics in the analysis of transport: general flow-force relationships and the linear domain. Method Enzymol 171: 397–445

Rottenberg H (1979) Non-equilibrium thermodynamics of energy conversion in bioenergetics. Biochim Biophys Acta 549: 225–253

Roufogalis BD, Villalobo A (1989) The $(Ca^{2+} + Mg^{2+})$-ATPase. Purification and reconstitution. In: Raess BU, Godfrey (eds) The red cell membrane. Humana Press, Clifton, New Jersey, pp 75–101

Schatzmann HJ (1989) The calcium pump of the surface membrane and of the sarcoplasmatic reticulum. Annu Rev Physiol 51: 473–485

Stucki JW (1980a) The thermodynamic-buffer enzymes. Eur J Biochem 109: 257–267

Stucki JW (1980b) The optimal efficiency and the economic degrees of coupling of oxidative phosphorylation. Eur J Biochem 109: 268–277

Tanford C (1984) The sarcoplasmatic-reticulum pump. Localization of free-energy transfer to discrete steps of the reaction cycle. FEBS Lett 166: 1–7

Villalobo A, Roufogalis BD (1986) Proton countertransport by the reconstituted erythrocyte Ca^{2+}-translocating ATPase: evidence using ionophoretic compounds. J Membr Biol 93: 249–258

Walz D (1990) Biothermokinetics of processes and energy conversion. Biochim Biophys Acta 1019: 171–224

Walz D, Caplan SR (1988) Energy coupling and thermokinetic balancing in enzyme kinetics. Microscopic reversibility and detailed balance revisited. Cell Biophys 12: 13–28

Westerhoff HV, Melandri BA, Venturoli G, Azzone GF, Kell DB (1984) A minimal hypothesis for membrane-linked free energy transduction. The role of independent small coupling units. Biochim Biophys Acta 763: 257–292

Westerhoff HV, Scholte BJ, Hellingwerf KJ (1979) Bacteriorhodopsin in liposomes. I. A description using irreversible thermodynamics. Biochim Biophys Acta 547: 544–560

Westerhoff HV, van Dam K (1987) Thermodynamics and control of biological free energy transduction. Elsevier, Amsterdam

Wolfram S (1991) Mathematica, 2nd edn. Addison-Wesley, Redwood City

Yu X, Carroll S, Rigaud J-L, Inesi G (1993) H^{+} countertransport and electrogenicity of the sarcoplasmic reticulum Ca^{2+} pump in reconstituted proteoliposomes. Biophys J 64: 1232–1242

Yu X, Hao L, Inesi G (1994) A p*K* change of acidic residues contributes to cation countertransport in the Ca-ATPase of sarcoplasmatic reticulum. J Biol Chem 269: 16565–16661

Yu X, Inesi G (1995) Variable stoichiometric efficiency of Ca^{2+} and Sr^{2+} transport by the sarcoplasmatic reticulum ATPase. J Biol Chem 270: 4361–4367