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A theoretical study of double-inhibitor-titration curves in free-energy-transducing networks

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The general relationship between the double-inhibitor-titration curves and the kinetic properties of pumps in a delocalized chemiosmotic free-energy-transducing network is studied. The kinetic conditions for a delocalized system to generate the observed double-inhibitor-titration results are derived and the effectiveness of double-inhibitor experiments in discriminating between the localized and the delocalized proton-coupling mechanisms is assessed. It is found that, using simple enzymatic cycles for the kinetics of the pumps in a delocalized network, one can reproduce the experimentally measured double-inhibitor-titration curves that were widely used to argue against the delocalized mechanism. This implies that double-inhibitor-titration curves alone are not sufficient to discriminate between localized and delocalized coupling systems. Additional information concerning the kinetic responses of isolated pumps on the proton gradient across the membrane and inhibitor concentrations are required.

Introduction

In Mitchell's chemiosmotic hypothesis [1,2] the H^+ electrochemical potential difference ($\Delta\tilde{\mu}_H$) of the two bulk phases across the membrane of the free-energy transduction systems acts as the coupling intermediate between the input (the redox or the light-driven) and the output (ATPase) H^+ pumps. The use of the 'bulk' phase H^+ electrochemical potential implies that protons are 'delocalized' such that the protons displaced by any redox pump can be used equally by all of the ATPase pumps in the system. Recently, several reports (see Ref. 3 and the references cited therein) have appeared to argue against this 'delocalized' proton hypothesis in favor of the so-called 'localized' small coupling unit concept. That is, instead of a common H^+ pool, the protons pumped by an input pump form a localized H^+ pool which can be used exclusively by only one or a few output pumps of the system. Among the evidences used to argue against the delocalized view are the results of the 'double-inhibitor-titration' (DIT) experiments [4–13]. In these experiments, the steady-state flux of the output pump (V_o) is titrated with the inhibitor of the input pump (I_i) or the catalyst (protonopher) of the leak (I_l), in the presence and absence of inhibitors of the output pump (I_o). The general idea behind this type of experiment is as follows. In a delocalized proton-coupling system, the output pump becomes more and the input pump less rate limiting when I_o is present. Thus, the effect of I_i on the output flux (V_o) should decrease when I_o is present in the system. As a result, when normalized, the titration curve of V_o vs. I_i in the presence of I_o should lie

Abbreviation: P_i, inorganic phosphate.

above the one without I_o. In other words, the slope of the nromalized titration curve at $I_i = 0$ should decrease as I_o is increased (see Fig. 1b). But, as shown in Fig. 1b, results opposite to this expectation are obtained in real systems [4–13]. That is, instead of less control, the input pump seems to be more effective in controlling the flux of the output pump when inhibitors of the latter are present. Similar results have also been found in titrating the flux of the output pump with protonophores. These results have been interpreted as evidence for the breakdown of the delocalized chemiosmotic hypothesis [3–11].

The above rationale of the double-inhibitor-titration experiments seems intuitively reasonable. Yet, it has been argued (e.g. Refs. 7,10 and 14–17) that under certain conditions a delocalized proton-coupling system might give results opposite to the intuitive expectation described above. For example, Pietrobon and Caplan [15] showed that when the proton fluxes of the input and the output pumps were linear functions of $\Delta\tilde{\mu}_H$ the titration with uncouplers followed the experimental DIT results. Davenport [16] also obtained the same results based on highly approximated flux functions. Using liposomes reconstituted with bacteriorhodopsin and ATP synthase molecules (presumed to be a delocalized system), Van der Bend et al. [17] showed that expected DIT results for delocalized systems were obtained only for uncouplers, not for input inhibitors. These results imply that the DIT curves may not be as useful as originally thought in discriminating between delocalized and localized models.

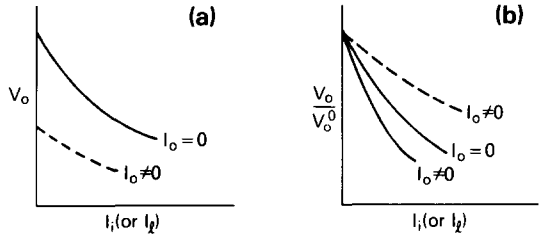

Fig. 1. (a) Expected double-inhibitor-titration (DIT) curves for delocalized systems. V_o is the flux of the output pump (ATP synthesis). I_i, I_o and I_l are the concentrations of inhibitors of the input, the output, and the leak pumps, respectively. (b) Normalized DIT curves. The dashed curve is the one expected intuitively for the delocalized system. The solid curve with $I_o \neq 0$ is the one found experimentally [4,5].

A clear assessment on the value of DIT results as assays for the extent of (de)localization of the coupling intermediates in an energy-coupling network has been lacking for some time, because the problem is inherently complex. For example, upon partial inhibition of one of the pumps in the system, the concentration of the coupling intermediate changes, and this may affect the kinetics of all enzymes in the system (including the partially inhibited pump). That is, the extent of inhibition of enzyme activities is a property of the entire network rather than of the inhibited enzyme alone.

One way to analyze the systemic properties of energy metabolism, is the Metabolic Control Theory of Kacser and Burns [18] and Heinrich and Rapoport [19] (for a review, see Ref. 20). Such an analysis relates the effect of inhibitors on fluxes to enzyme properties and flux ratios and is very useful in studying DIT experiments. This line of approach has been studied by Westerhoff and Kell [21]. In this paper, a different approach based on direct functional analysis will be presented. At first, we derive the relationship between the DIT curves and the kinetic properties of pumps in the network. In particular, we examine the condition(s) for a delocalized coupling system to have the observed DIT results. Then we show that these conditions can be satisfied easily for systems with cyclic enzymatic pump kinetics. That is, the experimental DIT curves can be obtained for delocalized systems with pumps described by enzyme cycles. The use of enzyme cycles makes the present analysis more general than those by Pietrobon and Caplan [15] and by Davenport [16].

General formula for testing double-inhibitor titrations

In this section, the general relation between DIT curves and the kinetic properties of individual (isolated) pumps in a delocalized energy-transducing system will be investigated. In particular, we will focus on the conditions that the system would be able to generate the observed DIT results.

We consider a vesicular membrane system con-

sisting of three kinds of pump: (1) the input (electron-transfer chain); (2) the output (ATP synthase); and (3) the leak (see Fig. 2). The input pumps use the free energy of electron-transfer chains (ΔG_i) to pump protons from the outisde phase of the vesicle to the inside phase. The output pumps use the electrochemical potential of protons thus generated to overcome the free-energy difference between ADP plus P_i and ATP (ΔG_o) in the system to synthesize ATP from ADP and P_i. In other words, in the input pumps the inward proton flux (J_i) is coupled to the redox flux (V_i) of the electron-transfer chains and in the output pumps the outward proton flux (J_o) is coupled to the flux of ATP synthesis (V_o). The leak process is a passive reaction which allows passage of protons through membrane down the proton electrochemical gradient across the membrane.

For simplicity, we assume that there is no electrical potential across the membrane (but see Discussion) so that the proton fluxes of the pumps will depend only on the H^+ concentration gradient of the system. The concentration of H^+ outside the system (c_1) is assumed to be buffered so that c_1 is a constant. Then, at constant ΔG_i and ΔG_o and in the presence of specific inhibitors, the total proton flux of each pump is a function of (i) the concentration of protons in the inside phase (c) and (ii) the concentrations of specific inhibitors (or activator as in J_l) present in the system:

$$J_i = J_i\,(n_i, c, I_i) \tag{1a}$$

$$J_o = J_o\,(n_o, c, I_o) \tag{1b}$$

$$J_l = J_l\,(n_l, c, I_l) \tag{1c}$$

where n_i, n_o and n_l are the total number of the input, the output, and the leak pumps in the absence of inhibitors, respectively. The sign of the flux is assigned positive when it flows from the inside to the outside. Thus, for systems in Fig. 2, J_i will be negative, while J_o and J_l will be positive. The steady state of the system (at constant ΔG_i and ΔG_o) is reached when the net transport of protons to the inside (or outside) is zero:

$$J_i + J_o + J_l = 0 \tag{2}$$

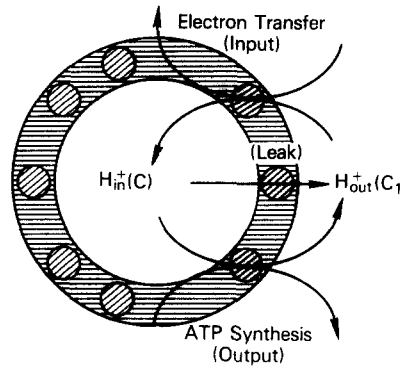

Fig. 2. Delocalized proton-mediated free-energy-transducing network. The pumps are represented by spheres. The input pumps transport protons from the outside phase to the inside phase, using the free energy of the electron-transfer chains. The output pumps use the proton gradient thus generated to synthesize ATP. The protons pumped by one pump can be used by any of the other pumps (delocalized system).

Eqn. 2 can be used to obtain the steady-state proton concentration of the inside phase c^{ss} as a function of the concentrations of inhibitors:

$$c^{ss} = f(I_i, I_o, I_l) \tag{3}$$

Thus, at steady-state the concentration of protons inside the vesicle is not an independent variable. The only independent variables of the system at constant ΔG_i and ΔG_o are I_i, I_o and I_l.

Similar to the proton fluxes, both V_i and V_o are also functions of c, I_i and I_o:

$$V_i = V_i(n_i, c, I_i);\ V_o = V_o(n_o, c, I_o) \tag{4}$$

In general, V_i and V_o can be related to J_i and J_o as:

$$V_i(n_i, c, I_i) = \gamma_i(c, I_i) J_i(n_i, c, I_i) \tag{5a}$$

$$V_o(n_o, c, I_o) = \gamma_o(c, I_o) J_o(n_o, c, I_o) \tag{5b}$$

where the γ's are the 'coupling stoichiometries' of the pumps. For example, if two protons have to be pumped across the output pump in order to produce one molecule of ATP, γ_o is equal to 1/2. For a 'perfect-coupled' pump, the value of γ is a constant independent of c and I. In this case, V_i (V_o) is always proportional to J_i (J_o). On the other hand, if the coupling is not perfect, the so-called 'slipping' occurs and the ratio of V_i/J_i becomes a

function of c and I_i.

As in DIT experiments, we will study the titration curves of the steady-state ATP output fluxes $(V_o)_{ss}$ as a functin of the concentration of inhibitors of the input pump (I_i) or activator of the leak pump (I_l) and investigate the changes in the initial slope of the titration curves when I_o is added to the system. The initial slope of the normalized titration curve of the ATP output flux V_o (we drop the subscripts ss from now on, for simplicity) with respect to the input inhibitor I_i is defined as

$$S_i^o = \left\{ \frac{1}{V_o} \frac{\partial V_o}{\partial I_i} \right\}_{I_l=0} = \left\{ \frac{1}{V_o} \frac{\partial V_o}{\partial c} \frac{\partial c}{\partial I_i} \right\}_{I_l=0} \tag{6}$$

Similarly, the slope of the normalized titration curve of V_o with respect to I_l (at steady state) is defined as

$$S_l^o = \left\{ \frac{1}{V_o} \frac{\partial V_o}{\partial I_l} \right\}_{I_l=0} = \left\{ \frac{1}{V_o} \frac{\partial V_o}{\partial c} \frac{\partial c}{\partial I_i} \right\}_{I_l=0} \tag{7}$$

One must note that the value of $\partial V_o/\partial c$ can be obtained experimentally by studying the functional dependence of V_o on c of an isolated output pump. In contrast, the value of $\partial c/\partial I_i$ has to be obtained by studying the entire energy-transducing system. However, if we differentiate Eqn. 2 with respect to the concentration of the inhibitor (or activator) I_α, we get

$$\frac{\partial J_\alpha}{\partial I_\alpha} + \frac{\partial J_i}{\partial c} \frac{\partial c}{\partial I_\alpha} + \frac{\partial J_o}{\partial c} \frac{\partial c}{\partial I_\alpha} + \frac{\partial J_l}{\partial c} \frac{\partial c}{\partial I_\alpha} = 0, \qquad \alpha = i, o, l \tag{8}$$

Thus

$$\frac{\partial c}{\partial I_\alpha} = -\frac{1}{X} \frac{\partial J_\alpha}{\partial I_\alpha} \tag{9}$$

where

$$X \equiv \frac{\partial J_i}{\partial c} + \frac{\partial J_o}{\partial c} + \frac{\partial J_l}{\partial c} \tag{10}$$

Substituting Eqn. 9 into Eqns 6 and 7, we get

$$S_i^o = -\left\{ \frac{1}{XV_o} \left(\frac{\partial V_o}{\partial c} \right) \left(\frac{\partial J_i}{\partial I_i} \right) \right\}_{I_l=0} \tag{11a}$$

$$S_l^o = -\left\{ \frac{1}{XV_o} \left(\frac{\partial V_o}{\partial c} \right) \left(\frac{\partial J_l}{\partial I_l} \right) \right\}_{I_l=0} \tag{11b}$$

These equations relate the slopes of the titration curves to the kinetic properties of the individual pumps. That is, although the slopes of the titration curves are properties of the entire system, they can be obtained from the kinetic properties of the isolated pumps.

Since the proton flux of each pump increases monotonically as the value of c is increased, the values of $\partial J_i/\partial c$, $\partial J_o/\partial J$ and $\partial J_l/\partial c$ are always positive no matter what the c value is. As a result, the X in Eqn. 10 is also always positive. Similarly, $\partial V_o/\partial c$ will also be a positive quantity. At steady state, J_i is negative. Thus, $\partial J_i/\partial I_i$ will be a positive quantity because I_i is an inhibitor. Similarly, $\partial J_l/\partial I_l$ will also be positive, because J_l at steady state is positive and I_l is an activator (protonophere). As a result, both S_i^o and S_l^o in Eqns. 11 and 12 are negative quantities consistent with those negative slopes observed in real systems (see Fig. 1).

The experimental DIT results show that both S_i^o and S_l^o become more negative when I_o in the system is increased (see Fig. 1b). In order for a delocalized system to show this kind of behavior, the differentials of S_i^o and S_i^l with respect to I_o have to be negative at $I_o = 0$:

$$\left(\frac{\partial S_i^o}{\partial I_o} \right)_{I_o=0} < 0; \quad \left(\frac{\partial S_l^o}{\partial I_o} \right)_{I_o=0} < 0 \tag{12}$$

Thus, from Eqns. 11 and 12, we obtain the conditions for the system to have the observed DIT result:

$$\left(\frac{\partial J_\alpha}{\partial I_\alpha} \right)_{I_\alpha=0} \cdot \left\{ \frac{\partial J_o}{\partial I_o} \cdot \left[\gamma_o \frac{\partial J_o}{\partial c} + J_o \frac{\partial \gamma_o}{\partial c} \right]^2 + \gamma_o J_o \left[\frac{\partial J_o}{\partial I_o} \frac{Y}{X} - \frac{\partial^2 J_o}{\partial c \partial I_o} \right] \cdot \left[\gamma_o \frac{\partial J_o}{\partial c} + J_o \frac{\partial \gamma_o}{\partial c} \right] + X \cdot \left[\gamma_o^2 J_o \frac{\partial^2 J_o}{\partial c \partial I_o} + \gamma_o J_o^2 \frac{\partial^2 \gamma_o}{\partial c^2} - \gamma_o^2 \frac{\partial J_o}{\partial c} \frac{\partial J_o}{\partial I_o} \right.$$

$$\left. - J_o^2 \frac{\partial \gamma_o}{\partial c} \frac{\partial \gamma_o}{\partial I_o} \right] - \gamma_o J_o \frac{\partial J_o}{\partial I_o} \cdot \left[\gamma_o \frac{\partial^2 J_o}{\partial c^2} + 2 \frac{\partial \gamma_o}{\partial c} \frac{\partial J_o}{\partial c} + J_o \frac{\partial^2 \gamma_o}{\partial c^2} \right] \Bigg\}_{I=0} - \frac{\partial^2 J_\alpha}{\partial c \partial I_\alpha}_{I_\alpha=0} \cdot \left\{ \gamma_o J_o \frac{\partial J_o}{\partial c} \cdot \left[\gamma_o \frac{\partial J_o}{\partial c} + J_o \frac{\partial \gamma_o}{\partial c} \right] \right\}_{I=0} > 0$$

$$(\alpha = i, l) \tag{13}$$

where $I = 0$ means $I_i = I_o = I_1 = 0$ and

$$Y \equiv \frac{\partial^2 J_i}{\partial c^2} + \frac{\partial^2 J_o}{\partial c^2} + \frac{\partial^2 J_1}{\partial c^2}, \tag{14}$$

This is the general condition for a delocalized proton coupling system to generate the experimental DIT results. Any delocalized system that show DIT results must obey this inequality. Again we like to emphasize that every term in Eqns. 13 and 14 can be evaluated experimental from the isolated pumps in vitro. Thus, when the kinetic properties of isolated pumps are available, Eqn. 13 may be used to differentiate between localized and delocalized models. For example, if the pumps do not obey the kinetic condition in Eqn. 13, then the protons in the system are definitely not delocalized.

If the coupling between the proton flux and the ATP flux in the output pump is perfect, γ_o is constant and Eqn. 13 can be simplified as

$$\left(\frac{\partial I_\alpha}{\partial I_\alpha}\right)_{I_\alpha = 0} \cdot \left\{ \left[X - \frac{\partial J_o}{\partial c} \right] \cdot \left[J_o \frac{\partial^2 J_o}{\partial c \partial I_o} - \frac{\partial J_o}{\partial c} \frac{\partial J_o}{\partial I_o} \right] + J_o \frac{\partial J_o}{\partial I_o} \cdot \left[\frac{\partial J_o}{\partial c} \frac{Y}{X} - \frac{\partial^2 J_o}{\partial c^2} \right] \right\}_{I=0} - \left(\frac{\partial^2 J_\alpha}{\partial c \partial I_\alpha}\right)_{I_\alpha = 0} \cdot \left[J_o \frac{\partial J_o}{\partial c} \frac{\partial J_o}{\partial I_o} \right]_{I=0} > 0 \tag{15}$$

Eqns. 13 and 15 are applicable to delocalized systems without specifying the kinetic mechanism of the pumps. However, if the reactions between inhibitors and enzymes follow the irreversible inhibition mechanism, the conditions in Eqns. 13 and 15 can be simplified as will be shown in the next section.

Irreversible inhibition mechanism

An irreversible inhibitor binding means that enzymes lost their activities irreversibly when bound with inhibitors. In this case, the total enzyme activity in the system is equal to the number of enzymes that are not bound with inhibitors. Also, the kinetic property of an enzyme that is free from inhibitor binding is not affected by the presence of inhibitors in the system. Thus, let $j_i(c)$ be the proton flux through a single input pump at c in the absence of I_i, then the total input proton flux in Eqn. 1a can be expressed as:

$$J_i = (n_i - I_i)\, j_i(c) \tag{16}$$

where n_i is the total number of input pumps originally in the system and I_i is the concentration of inhibitors. Similarly, the total proton flux of the output pumps can be expressed as:

$$J_o = (n_o - I_o)\, j_o(c) \tag{17}$$

In contrast to the binding of inhibitors to the input and the output pumps, the binding of protonopheres (uncouplers) is assumed to be reversible. Also, the channels (or pathways) created by the bound protonopheres are assumed to have the same kinetic properties as those of the original leak process. Thus, let K_1 be the equilibrium binding constant of protonopheres (I_1) with the membrane, then

$$J_1 = (n_1 + K_1 I_1)\, j_1(c) \tag{18}$$

where n_1 is the number of original leak pumps in the membrane.

When substituted with Eqns. 16–18, the general conditions for DIT results in Eqn. 13 becomes

$$-j_i \cdot A_o > \frac{\partial j_i}{\partial c} \cdot v_o \qquad \text{(titration with } I_i\text{)} \tag{19a}$$

$$j_1 \cdot A_o > -\frac{\partial j_1}{\partial c} \cdot v_o \qquad \text{(titration with } I_1\text{)} \tag{19b}$$

where v_o and A_o are defined as

$$v_o \equiv \gamma_o \left[\gamma_o \frac{\partial j_o}{\partial c} + j_o \frac{\partial \gamma_o}{\partial c} \right] \tag{20}$$

$$A_o \equiv \gamma_o \left[\gamma_o \frac{\partial^2 j_o}{\partial c^2} + j_o \frac{\partial^2 \gamma_o}{\partial c} \right] + \gamma_o \frac{\partial j_o}{\partial c} \frac{\partial \gamma_o}{\partial c} - j_o \frac{\partial \gamma_0^2}{\partial c} - \frac{v_o Y_o}{X_o} \tag{21}$$

The X_o and Y_o in Eqns. 20 and 21 are the X and Y values evaluated at $I = 0$.

For perfect-coupled output pumps, γ_o is inde-

pendent of c and

$$v_o = \gamma_o^2 \frac{\partial j_o}{\partial c} \tag{22}$$

$$A_o = \gamma_o^2 \frac{\partial^2 j_o}{\partial c^2} - \gamma_o^2 \frac{\partial j_0}{\partial c} \frac{Y_o}{X_o} \tag{23}$$

Thus, the DIT conditions in Eqn. 19 become

$$-j_i \left[\frac{\partial^2 j_o}{\partial c^2} - \frac{\partial j_o}{\partial c} \frac{Y_o}{X_o} \right] > \frac{\partial j_i}{\partial c} \frac{\partial j_o}{\partial c} \tag{24a}$$

$$j_1 \left[\frac{\partial^2 j_o}{\partial c^2} - \frac{\partial j_o}{\partial c} \frac{Y_o}{X_o} \right] > -\frac{\partial j_1}{\partial c} \frac{\partial j_o}{\partial c} \tag{24b}$$

One must note that, at steady-state, j_i is negative and j_1 is positive. Also, $\partial j_i/\partial c$, $\partial j_o/\partial c$, and $\partial j_1/\partial c$ are all positive quantities. Thus, if Eqn. 24a is true, Eqn. 24b will be true automatically. In other words, if the titration with I_i shows DIT results, so will the titration with protonopheres (I_1). The reverse is not true.

When j_i, j_o and j_1 are linear functions of c, the second-order differentials, $\partial^2 j/\partial c^2$, etc., will be equal to zero. As a result, $Y_o = 0$. Then the condition in Eqn. 24a can never be fulfilled. This means that no experimental DIT behavior will be observed in the I_i titration curve. On the other hand, Eqn. 24b is always true in this case, because the right-hand side of Eqn. 24b is always negative. As a result, titration with I_1 (protonopheres) will always give the DIT results.

Illustrative model calculations

In previous sections, we obtained the kinetic conditions that the pumps in a delocalized model have to follow in order to generate the observed DIT results. Thus, if the kinetic properties of the pumps (such as $\partial j_i/\partial c_1$, $\partial^2 j_i/\partial c^2$, etc.) are available, a discrimination between localized and delocalized mechanisms may be possible based on these conditions and the observed DIT results. In this section, we show that arbitrary pump models are readily obtainable that would satisfy these conditions. In other words, the observed DIT results can be derived also from the delocalized system. For simplicity, only perfect-coupled pumps are considered here.

Irreversible-inhibition case

We need explicit expressions for $j_i(c)$, $j_o(c)$ and $j_1(c)$ in order to test the DIT contraint in Eqns. 24a and 24b and to calculate the DIT curves. In general, the kinetics of an energy-coupling pump is very complicated in that it contains many enzyme states and many enzymatic reaction steps. For simplicity, we will examine a very simple case that the enzymatic kinetics of both the input and the output pumps will be represented by a three-state cycle and the leak by a two-state cycle as shown in Fig. 3. The input pump couples the transport of H^+ to electron transfer. For each clockwise turnover, three protons from the outside are pumped into the inside of the vesicle in the expense of the free energy dissipated in the electron-transfer chain. The output pump, which couples the transport of H^+ to ATP synthesis, transfers two protons from the inside to the outside per counterclock wise turnover. The leakage (l) is a passive enzymatic reaction with one proton exchanged each turnover.

In each diagram, the steps where protons are picked up and released by the enzyme are shown explicitly, while the steps where reactions of electron-transfer chain and ATP synthesis occur are not. Thus, the rate constants in each diagram must be related to the pump's thermodynamic force [22] by

$$\frac{k_{13}^i k_{32}^i k_{21}^i}{k_{31}^i k_{23}^i k_{12}^i} = e^{\Delta G_i/RT} \tag{25}$$

$$\frac{k_{13}^o k_{32}^o k_{21}^o}{k_{31}^o k_{23}^o k_{12}^o} = e^{\Delta G_o/RT} \tag{26}$$

$$\frac{\alpha_{12}\alpha_{21}}{\beta_{12}\beta_{21}} = 1 \tag{27}$$

where ΔG_i is the free-energy difference between the reduced and the oxidated states of the electron transfer chain and ΔG_o is the free-energy difference between ATP and ADP plus P_i. At any c value, the proton flux of an input pump can be written in terms of the rate constants [22] and the value of c_1 as

$$j_i(c) = \frac{3}{\Sigma}\left(k_{12}^i k_{23}^i k_{31}^i c^3 - k_{21}^i k_{32}^i k_{13}^i c_1^3\right) \tag{28}$$

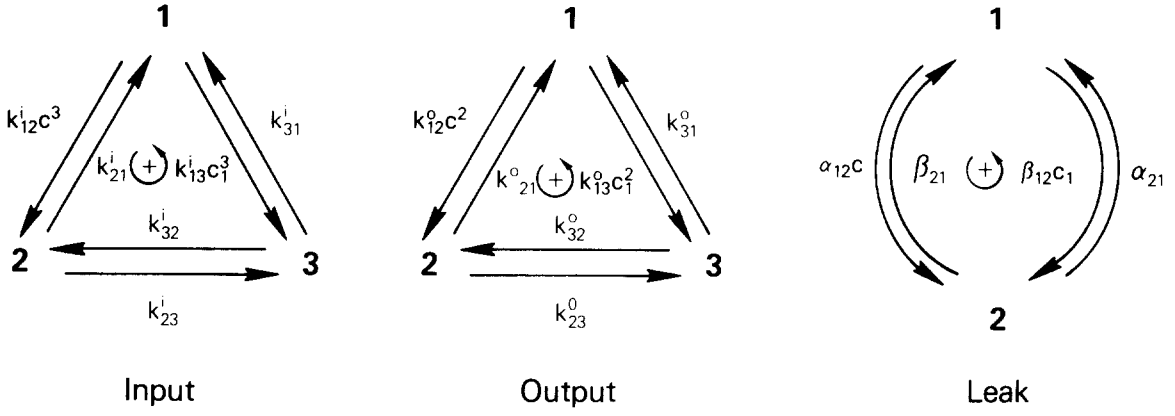

Fig. 3. The kinetic diagrams of the three pumps used in model calculations. The values of the rate constants used in the calculations are: (1) Input: $k^i_{21} = k^i_{31} = k^i_{32} = k^i_{23} = 1.0$; $k^i_{12} = 100.0$; $k^i_{13} = 10^{15}$; (2) output: $k^o_{21} = k^o_{31} = k^o_{32} = k^o_{23} = 1.0$; $k^o_{12} = 10^4$; $k^o_{13} = 0.298 \cdot 10^8$; (3) leak: $\alpha_{21} = \beta_{21} = 10^{-5}$; $\alpha_{12} = \beta_{12} = 2.0$.

where

$$\Sigma \equiv \left(k^i_{12}k^i_{32} + k^i_{12}k^i_{31} + k^i_{12}k^i_{23}\right)c^3 + k^i_{21}k^i_{31} + k^i_{32}k^i_{21} + k^i_{23}k^i_{31} + \left(k^i_{32} + k^i_{23} + k^i_{21}\right)k^i_{13}c_1^3. \quad (29)$$

Eqn. 28 contains six rate constants, five of them are independent parameters (Eqn. 25 eliminate one parameter). However, Eqn. 28 can be rearranged into a simpler canonical form containing only three parameters:

$$j_i(c) = 3\alpha_i \frac{c^3 - a_i}{c^3 + b_i} \quad (30)$$

where a_i, b_i and α_i are constants and defined as:

$$a_i \equiv \frac{k^i_{21}k^i_{32}k^i_{13}c_1^3}{k^i_{12}k^i_{23}k^i_{31}} \equiv c_1^3\, e^{\Delta G_i/RT} \quad (31)$$

$$b_i \equiv \frac{k^i_{21}k^i_{31} + k^i_{32}k^i_{21} + k^i_{23}k^i_{31} + \left(k^i_{32} + k^i_{23} + k^i_{21}\right)k^i_{13}c_1^3}{k^i_{12}\left(k^i_{32} + k^i_{31} + k^i_{23}\right)} \quad (32)$$

$$\alpha_i \equiv \frac{k^i_{23}k^i_{31}}{k^i_{32} + k^i_{31} + k^i_{23}} \quad (33)$$

The 3 in Eqns. 28 and 30 comes from the assumption that three protons are involved per turnover. Similar flux expressions can be obtained for the output and the leak pumps:

$$j_o(c) = 2\alpha_o \frac{c^2 - a_o}{c^2 + b_o} \quad (34)$$

$$j_l(c) = \alpha_l \frac{c - a_l}{c + b_l} \quad (35)$$

where a_o, b_o, and α_o are the same as those in Eqns. 31–33 for a_i, b_i and α_i, except that the superscript (and the subscript) i is changed to o. The a_l, b_l, and α_l are defined as:

$$a_l \equiv \frac{\beta_{12}\beta_{21}c_1}{\alpha_{12}\alpha_{21}} = c_1 \quad (36)$$

$$b_l \equiv \frac{\alpha_{21} + \beta_{21} + \beta_{12}c_1}{\alpha_{12}} \quad (37)$$

$$\alpha_l \equiv \alpha_{21} \quad (38)$$

As shown in Eqn. 31, a_i is a constant because c_1 and ΔG_i are constants. So are a_o and a_l. Thus, in this particular model, the adjustable parameters are b_i, α_i, etc. With explicit expressions for the steady-state proton fluxes given in Eqns. 30, 34 and 35, the search for models that satisfy the condition in Eqns. 24a and 24b (and therefore show DIT behavior) can be carried out easily. Table I lists the values of the parameters (b_i, α_i, etc.) of a model found to have satisfied the DIT condition (Eqn. 24). In this particular model, the

TABLE I

KINETIC PARAMETERS USED IN THE CALCULATION OF THE DIT CURVES IN FIGS. 4 AND 6

	a	b	α	n	J^o	$\frac{\partial J}{\partial c}$	$\frac{\partial^2 j}{\partial c^2}$
Input pump (i)	10^{-8}	10^{-2}	$\frac{1}{3}$	10^{6}	-1.0	$3\cdot10^{-8}$	$6\cdot10^{-3}$
Output pump (o)	$0.298\cdot10^{-10}$	10^{-4}	$\frac{1}{3}$	$1.7097\cdot10^{6}$	0.8	0.1333	$1.3333\cdot10^{4}$
Leak pump (l)	10^{-7}	10^{-5}	10^{5}	$4.04\cdot10^{4}$	0.2	0.2525	$-2.525\cdot10^{4}$

values of ΔG_i, ΔG_o and c_1 are set to $30RT$, $8RT$ and pH 7 respectively. The steady-state proton concentration inside the vesicle in the absence of inhibitors is set to pH 5 ($c_o = 10^{-5}$ M). The J^o values in the table represent the total proton fluxes of the three pumps at c_0 (without any inhibitors) and are set to -1, 0.8, and 0.2 for the input, the output and the leak respectively. The total number of the input pumps in the system (n_i) can be calculated from Eqn. 16 and is also shown in the table, along with n_o and n_l. One must note that the values of a_m, b_m and α_m (m = i, o, l) are not completely independent of each other, but related through the rate constants of the diagram (see Eqns. 31–33 and 36–38). The values of the rate constants that give the a, b, and α values in Table I are listed in the legend of Fig. 3.

In order to confirm that the experimental DIT curves can be reproduced by the model, the DIT curves of the model (Table I) were calculated both in the absence of I_o and at $I_o = 300$. The calculated inhibitor titration curves are normalized and plotted in Fig. 4. As shown in the figure, the experimental DIT behavior indeed is reproduced in both the input pump inhibition and the leak inhibition titrations.

Similar results were obtained when c_1 and c_0 are increased ten times larger (pH 6 and pH 4, respectively). We have also varied the proton stoichiometry of the pumps from 3 : 2 : 1 to 4 : 3 : 1 and obtained the same result. Thus, it seems rather easy to obtain the experimental DIT results in delocalized proton models.

Competitive inhibition case

Competitive inhibition means that inhibitors are competing with substrates for the same binding sites on enzymes. In other words, an extra state representing the inhibitor-enzyme complex has to be added to the enzymatic cycle diagram (see Fig. 5). Using the diagram method of Hill [9], the proton flux of the input pump shown in Fig. 5

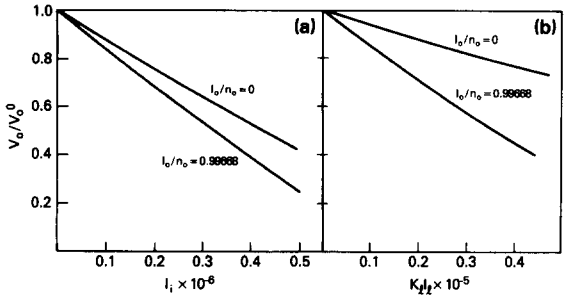

Fig. 4. Calculated double-inhibitor-titration curves for a delocalized system with irreversible inhibition mechanism. The kinetic parameters used in the calculation are shown in Table I.

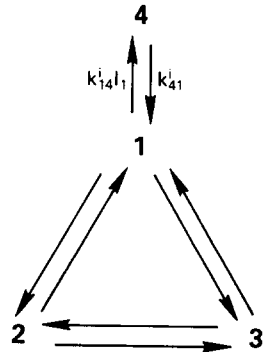

Fig. 5. The kinetic diagram of reversible inhibitor binding.

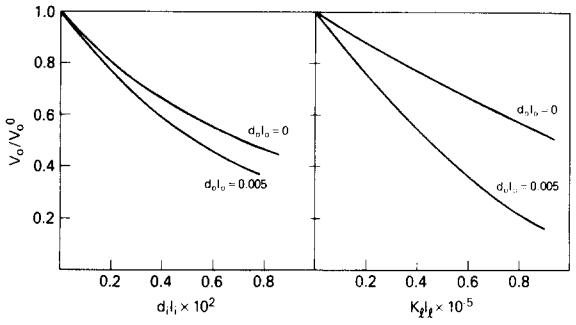

Fig. 6. Calculated DIT curves for a delocalized system with reversible inhibitor-binding mechanism.

can be shown to equal to

$$J_i(c) = 3n_i \frac{c^3 - a_i}{c^3 + b_i + d_i I_i} \tag{39}$$

were a_i and b_i are the same as those in Eqns. 31 and 32 and d_i is defined as:

$$d_i \equiv \frac{k_{14}^i \left(k_{32}^i k_{21}^i + k_{31}^i k_{21}^i + k_{23}^i k_{31}^i\right)}{k_{41}^i k_{12}^i \left(k_{32}^i + k_{31}^i + k_{23}^i\right)} \tag{40}$$

A similar expression can be obtained for the output pump:

$$J_o(c) = 2n_o \frac{c^2 - a_o}{c^2 + b_o + d_o I_o}. \tag{41}$$

Unlike the irreversible-inhibition case, the total proton fluxes passing through the input and the output pumps in this competitive inhibitor case cannot be separated into inhibitor-dependent and inhibitor-independent parts (compare Eqns. 39 and 41 with Eqns. 16 and 17). Thus, one has to use the general formalism shown in Eqn. 15 to test the DIT behavior. The test will be more difficult to carry out because more functions are involved. Therefore, instead of searching for new models, we simply used the same set of a_m, b_m and α_m (m = i, o, l) shown in Tables I and directly calculated the double titration curves for this competitive-inhibition case. The results are shown in Fig. 6. As can be seen from the figures, the experimentally observed DIT behavior is also obtained.

Discussion

The main purpose of this study was to investigate whether the DIT (dual-inhibitor titration) results alone would really rule out the delocalized proton-coupling mechanism in membrane free-energy transducing systems. We started with the derivation of the general condition that the kinetics of the pumps must follow in order to produce the observed DIT curves in a delocalized proton-coupling system. Then, using the derived conditions as guide, we searched and found some simple kinetic models that were able to reproduce the same DIT behavior as observed experimentally (Figs. 4 and 6). Thus, we conclude: without any information on the kinetics of the pumps the experimental DIT curves alone cannot be used as an unequivocal argument against the delocalized proton hypothesis. As discussed before, pietrobon and Caplan [15] and Davenport [16] have also obtained the same conclusion based on highly approximate flux-force functions. By using the enzyme cycle formulation, the treatment presented here is therefore more general.

We like to point out that, in our model calculations, the kinetics of the input and the output pumps have been described by simple three-state cycle diagrams. This is certainly an oversimplification: the transport of protons across energy-transducing membranes in oxidative phosphorylation (or photophosphorylation) system should be much more complicated than that. However, this simplification does not invalidate the general conclusion mentioned above, because complex diagrams are always reducible to simpler ones by assuming fast equilibrium between states or by removing the transient intermediate states from the diagrams [22]. In other words, a complex diagram can always be reduced to a three-state diagram if no constraint is applied to the values of the rate constants of the model.

As discussed in previous sections, the electrical potential part of the electrochemical gradient of H^+ has been neglected for simplicity in this study. That is, the membrane potential is assumed to be zero and the main contribution to the proton-motive force of the system comes from the pH gradient only. Thus, the analysis presented in this paper is in principle directly applicable only to systems

where membrane potential is unimportant, such as in chloroplast or in mitochondria and bacteria in the presence of ionopheres. However, the present analysis can be extended to include the membrane potential as an additional parameter. In this case, some of the rate constants of the pumps become membrane-potential dependent.

We like to emphasize that, although the DIT curves alone may not be able to dispute the delocalized proton hypothesis, DIT results coupled with kinetic informations of isolated pumps may be useful in differentiating between delocalized and localized systems. For example, if the in vitro kinetic properties of the input and the output pumps satisfy the DIT condition in Eqn. 13 (or in Eqn. 15 if the coupling between ATP flux and proton flux is perfect; or in Eqn. 24 if the inhibitor binding is irreversible), the protons in energy-transducing systems may be either localized or delocalized. In contrast, if the condition in Eqn. 13 is not satisfied, the protons are definitely not delocalized. This points out the importance of studying the kinetic properties of isolated pumps.

Since the equation may be useful in future application, it seems useful to discuss briefly what kind of kinetic measurements does Eqn. 13 require. As $\partial J_o/\partial c$ and $\partial^2 J_o/\partial c \partial_o$, etc. are the (partial) derivatives of J_o (the output proton flux) with respect to c (the proton concentration) and I_o (the inhibitor concentration), they can be evaluated directly from the function $J_o(c, I_o)$. Thus, the basic kinetic information Eqn. 13 needs are the three proton flux functions, $J_i(c, I_i)$, $J_o(c, I_o)$ and $J_l(c, I_l)$ plus the ATP flux function of the output pump, $V_o(c, I_o)$, (in order to evaluate γ_o, see Eqn. 5a). In principle, all the J's and the V_o can be obtained if the pumps can be isolated and reconstituted into lipid bilayer membranes. For example, the procedure to evaluate the $J_i(c, I_i)$ using reconstituted cytochrome oxidase can be summarized as follows: (1) keep the redox potential and the $[H^+]$ on the side where cytochrome oxidase draws the protons at constant; (2) vary the $[H^+]$ of the other side and the inhibitor concentration I_i and measure the proton flux pumped by the cytochrome oxidase; (3) repeat step 2 until the entire $J_i(c, I_i)$ function is obtained. In summary, lathough Eqn. 13 looks complicated, the kinetic information required to use this equation is not difficult to obtain in principle.

In the DIT experiments carried out by Kell and his colleagues [4,5], the V_o/V_i (ATP/redox) flux ratios of the system were found to be constant when titrated with inhibitors of the input pumps. In the calculations presented in Figs. 4 and 6 the ratios of the steady-state fluxes of the input pumps to those of the output pumps were found always to vary as the inhibitor concentrations of the input pumps were increased. Attempts to find parameters that generate both the DIT curves and a constant V_o/V_i flux ratio for three-state diagrams were failed. Currently, models with more complicated diagrams and models with 'slipping' pumps are being studied for this aspect. If this turns out to be negative also, then DIT curves coupled with a constant V_o/V_i flux ratio may be used to differentiate between 'localized' and 'delocalized' coupling mechanism.

In conclusion, this paper is not meant to argue against the 'localized' proton hypothesis proposed recently by a number of people, nor to discredit the usefulness of the DIT experiment, but to point out the fact that DIT curves alone are not enough to rule out the 'delocalized' view of Mitchill's chemiosmotic hypothesis. Kinetic properties of all three kinds of pumps have to be obtained before the discrimination between localized and delocalized proton models can be made based on double-inhibitor-titration curves.

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