Further comments on the logic of the application of uncoupler-inhibitor titrations for the elucidation of the mechanisms of energy coupling

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Following the appearance of two papers in this journal commenting on the logic of the application of uncoupler-inhibitor titrations as a means of discriminating between 'delocalized' and 'localized' chemiosmotic mechanisms [(1984) FEBS Lett. 176, 79–82; (1985) FEBS Lett. 186, 8–10], and in contrast with the arguments presented there and elsewhere, we show that in a linear model the increase in $\Delta\tilde{\mu}H$ which accompanies partial inhibition of the ATPases always leads to a relatively higher decrease of the rate of ATP synthesis by a given concentration of uncoupler in the presence of an ATPase inhibitor than in its absence. This is due to the fact that the same titre of uncoupler leads to a higher dissipative H^+ flow in the presence of inhibitor, since the driving force $\Delta\tilde{\mu}H$ is higher.

Uncoupler-inhibitor titration *Chemiosmotic hypothesis* *Localized vs delocalized mechanism* *Proton pump* *Oxidative phosphorylation* *Energy coupling*

1. INTRODUCTION

Two papers appeared recently in this journal [1,2] commenting on the logic of the application of a particular kind of double-inhibitor titration, the 'uncoupler-inhibitor' titration, introduced by Hitchens and Kell [3] as a means of discriminating between 'delocalized' chemiosmotic mechanisms and 'localized' mechanisms of energy transduction (reviews [4,5]). The uncoupler-inhibitor titration consists in measuring the stationary state rate of phosphorylation (or in general the rate of the $\Delta\tilde{\mu}H$-driven reaction) at different uncoupler concentrations in the presence or absence of an inhibitor of the ATPase (or in general of the secondary $\Delta\tilde{\mu}H$-utilizing proton pump). It has been found [3,6] that the relative inhibition of the rate of the energy-consuming reaction by a given concentration of uncoupler is greater in the presence of an inhibitor of the secondary pump than in its absence. This result has been considered inconsistent with the delocalized chemiosmotic model by the authors in [2–6], since in their opinion it can only be accounted for if the inhibited preparation has a lower $\Delta\tilde{\mu}H$ as compared with the uninhibited one. Instead, the delocalized chemiosmotic model predicts, and experiments show [7], that $\Delta\tilde{\mu}H$ in the presence of inhibition of the secondary pump is higher than in its absence.

The aim of this paper is to show that in contrast to the arguments in [2–6], the predicted and experimentally observed inhibitor-induced increase in $\Delta\tilde{\mu}H$ always leads, in a linear model of chemiosmotic energy coupling, to a relatively higher decrease of the rate of ATP synthesis by a given concentration of uncoupler in the presence of an ATPase inhibitor than in its absence. This is due to the fact that the same titre of uncoupler leads to a higher dissipative H^+ flow in the presence of inhibitor, since the driving force $\Delta\tilde{\mu}H$ is higher (and is not due, as argued in [1], to an inhibitor-induced decreased conductance of the ATPase as compared with that of the uncoupler; cf. [2]).

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2. SIMULATIONS OF UNCOUPLER-INHIBITOR TITRATIONS WITH A LINEAR MODEL OF CHEMIOSMOTIC ENERGY COUPLING

The simplest chemiosmotic protonic circuit is constituted by 3 elements in parallel: a $\Delta\tilde{\mu}$H-generating (i.e. a redox) proton pump, a $\Delta\tilde{\mu}$H-consuming (i.e. an ATPase) proton pump, and a 'leak', i.e. a pathway for passive diffusion of protons through the membrane. If, for the sake of argument, the pumps are assumed to be completely coupled and the relationships between flow and forces are assumed to be proportional, the proton flow through each element can be described by the following equations [8]:

$$J_H^e = n_e J_e = n_e L_e (A_e + n_e \Delta\tilde{\mu}H) \quad (1a)$$

$$J_H^P = n_P J_P = n_P L_P (A_P + n_P \Delta\tilde{\mu}H) \quad (1b)$$

$$J_H^l = L_H^l \Delta\tilde{\mu}H \quad (1c)$$

Here A_e and A_P are the positive affinities (the negative Gibbs free energy changes) of the redox and ATP hydrolysis reactions, respectively, n_e is the H^+/e^- stoichiometry of the redox pump, n_P is the H^+/ATP stoichiometry of the ATPase pump, J_e is the rate of electron transport, L_e is the proportionality coefficient between J_e and the driving force of the redox pump ($A_e + n_e\Delta\tilde{\mu}H$), J_P is the rate of ATP synthesis or hydrolysis ($J_P > 0$: hydrolysis, $J_P < 0$: synthesis), L_P is the proportionality coefficient between J_P and the driving force of the ATPase pump ($A_P + n_P\Delta\tilde{\mu}H$), and J_H^l is the flow of protons through the leak, while L_H^l is its conductance. L_e and L_P can be viewed as the conductance of the redox and ATPase pumps, respectively. In the stationary state of phosphorylation (state 3), the total proton flow is zero:

$$J_H^e + J_H^P + J_H^l = 0 \quad (2)$$

Note that J_H^e (in→out) is positive, J_H^P and J_H^l (out→in) are negative, and also $\Delta\tilde{\mu}H = \tilde{\mu}H^{in} - \tilde{\mu}H^{out}$ is negative. Substituting eqns 1a–c in eqn 2, the following expression for $\Delta\tilde{\mu}H$ in state 3 is easily derived:

$$\Delta\tilde{\mu}H = -(n_e L_e A_e + n_P L_P A_P)/(n_e^2 L_e + n_P^2 L_P + L_H^l) \quad (3)$$

Once certain constant values are assigned to A_e, A_P, and the conductances and stoichiometries of the proton pumps, the titration with uncoupler can be simulated by computing $\Delta\tilde{\mu}H$ at increasing values of L_H^l from eqn 3, and by substituting the computed values into eqn 1b to obtain J_P (the rate of ATP synthesis in state 3) at different uncoupler concentrations. Inhibition of the ATPase pumps by inhibitors such as oligomycin can be simulated by multiplying the coefficient L_P by the inhibition factor f_P, which varies from 1 at zero inhibitor concentration to 0 at a concentration of inhibitor which inhibits all the pumps.

The computation of $\Delta\tilde{\mu}H$ and J_P in state 3 as a function of L_H^l can be carried out at different values of f_P. Fig.1 shows 2 examples (differing in the values of L_e) of the computed normalized uncoupler titrations for $f_P = 1$ and $f_P = 0.1$. $J_P(0)$ is the rate of ATP synthesis at zero uncoupler concentration. Whatever the values assigned to the constant parameters in the equations, the computed result always follows the pattern shown in fig.1: the relative decrease in the rate of ATP synthesis induced by a given concentration of uncoupler is higher in the presence of an inhibitor of the ATPase than in its absence. The ratio of conductances of the 2 proton pumps L_e/L_P (or more precisely the ratio $n_e^2 L_e/n_P^2 L_P$) determines,

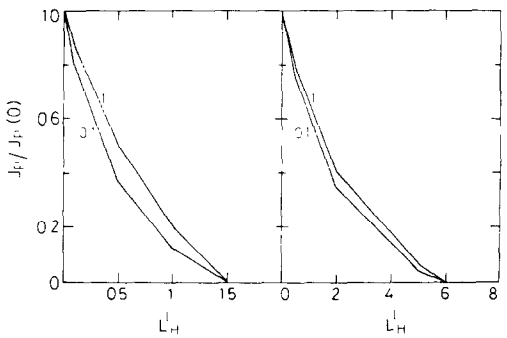

Fig.1. Relative variation of the rate of ATP synthesis as a function of the leak conductance, L_H^l, in the absence ($f_P = 1$) and presence ($f_P = 0.1$) of an ATPase inhibitor. $J_P(0)$ is the rate of ATP synthesis at zero L_H^l. Parameters used in the computation: $A_e = 4$, $A_P = 1$, $n_e = 1$, $n_P = 1$. Left: $L_e = 0.5$; $L_P = 1$; $L_H^l = 0.1, 0.5, 1, 1.5$; $J_P(0) = -1$ for $f_P = 1$; $J_P(0) = -0.25$ for $f_P = 0.1$. Right: $L_e = 2$; $L_P = 1$; $L_H^l = 0.5, 2, 5, 6$; $J_P(0) = -2$ for $f_P = 1$, $J_P(0) = -0.286$ for $f_P = 0.1$.

whatever the individual values of L_e and L_P, the difference in the titration curves with and without inhibitor. The lower the value of L_e/L_P, the larger the difference. Note that the concentration of uncoupler necessary to abolish ATP synthesis completely does not change in the presence of ATPase inhibitors. For a given A_e/A_P ratio the fully inhibitory titre of uncoupler is proportional to L_e:

$$(L_H^l)_{J_P=0} = n_e^2 L_e\left(\frac{n_P A_e}{n_e A_P} - 1\right) \quad (4)$$

It is clear that by introducing an inhibition factor f_e for the redox pumps analogous to f_P, all kinds of double inhibitor titrations [5,9] can be computed from eqns 1–3. A detailed analysis of these computations and of the influence of the ratio L_e/L_P on the relative inhibition of the rate of ATP synthesis and on the relative changes in $\Delta\tilde{\mu}H$ induced by a given combination of inhibitors is presented elsewhere [10].

3. DISCUSSION

The computation from eqns 1–3 of the uncoupler-inhibitor titrations shows that a delocalized chemiosmotic model of energy coupling with linear flow-force relationships predicts that the relative lowering of the rate of ATP synthesis by a given titre of uncoupler is greater in the presence of an ATPase inhibitor than in its absence. This conclusion is not intuitively obvious since the authors in [2–6] predicted the opposite. It becomes clear if one looks at the relative variations of $\Delta\tilde{\mu}H$ induced by a given concentration of uncoupler in the absence and presence of ATPase inhibitor. These are shown, together with the corresponding variations of J_P, in table 1, where $\Delta\tilde{\mu}H(0)$ is $\Delta\tilde{\mu}H$ in state 3 without uncoupler ($L_H^l = 0$). A given concentration of uncoupler gives rise to a relatively larger decrease of $\Delta\tilde{\mu}H$ in the presence of an ATPase inhibitor than in its absence. This relatively larger decrease in $\Delta\tilde{\mu}H$ is accompanied by a relatively larger decrease in the rate of ATP synthesis, since the 2 are proportional (as can be seen in table 1). The relatively larger decrease of $\Delta\tilde{\mu}H$ is due to the fact that in the presence of ATPase inhibitor the absolute value of $\Delta\tilde{\mu}H$ in state 3 is larger than in the absence of inhibitor. As a consequence the same uncoupler titre (L_H^l) brings

Table 1

Relative variations of $\Delta\tilde{\mu}H$ and J_P as a function of L_H^l with and without ATPase inhibitor

L_H	f_P	$\Delta\tilde{\mu}H/\Delta\tilde{\mu}H(0)$	$J_P/J_P(0)$
0.1	1	0.938	0.875
0.1	0.1	0.857	0.8
0.5	1	0.75	0.5
0.5	0.1	0.546	0.364
1.0	1	0.6	0.2
1.0	0.1	0.375	0.125
1.5	1	0.5	0
1.5	0.1	0.286	0

Parameters used in the computation are as in fig.1 (left). $\Delta\tilde{\mu}H(0)$ is $\Delta\tilde{\mu}H$ at zero L_H^l. $J_P(0)$ is the rate of ATP synthesis at zero L_H^l. When $f_P = 1$ $\Delta\tilde{\mu}H(0) = -2$; when $f_P = 0.1$ $\Delta\tilde{\mu}H(0) = -3.5$

about a higher dissipative proton flow in the presence of inhibitor since its driving force $\Delta\tilde{\mu}H$ is higher. The key fact for an understanding of the results of uncoupler-inhibitor titrations within a delocalized chemiosmotic model is the predicted and experimentally found [7] increase in $|\Delta\tilde{\mu}H|$ which accompanies inhibition of the $\Delta\tilde{\mu}H$-utilizing pumps. In [2–6] it has been implied or argued erroneously that such an increase in $|\Delta\tilde{\mu}H|$ would lead to the opposite result, i.e. to a relatively lower decrease of J_P in the presence of inhibitor. Hence the authors in [2,3] consider the case of a constant $\Delta\tilde{\mu}H$ with or without ATPase inhibitor, in order to illustrate the logic of the application of uncoupler-inhibitor titrations. In this case their reasoning is correct, i.e. each addition of uncoupler does not decrease $|\Delta\tilde{\mu}H|$ (and therefore the rate of ATP synthesis) more effectively in the presence of ATPase inhibitor than in its absence, and the dissipative proton flow is the same under both conditions. The relevant physical quantities are the flows, which are given in the linear non-equilibrium thermodynamic description by a conductance coefficient times a thermodynamic force. As correctly pointed out by Van der Bend and Herweijer [2], the mistake of O'Shea and Thelen [1] in analyzing the logic of these titrations was to forget the thermodynamic forces and to consider the flows J_P and J_H^l equivalent to the conductances L_P and L_H^l. On the other hand, the authors in [2–6]

made an analogous mistake in predicting that an increase in $|\Delta\tilde{\mu}H|$ upon inhibition should increase the titre of uncoupler necessary to inhibit J_P to a given extent, since they consider the dissipative proton flow as equivalent to the conductance L_H^l.

In contrast to the prediction of the linear delocalized chemiosmotic model discussed above, Hitchens and Kell [3] found that in bacterial chromatophores the titre of uncoupler necessary to reach full inhibition was also decreased in the presence of venturicidin or DCCD. This result, however, has been criticized on experimental grounds in [11] and does not appear in [6].

Mills [12] has simulated uncoupler-inhibitor titrations using a simple non-linear model of chemiosmotic coupling and obtained results very similar to those of fig.1. Recently, Pietrobon and Caplan [13] studied the non-linear flow-force relationships for a 6-state proton pump model. By linking in parallel, through their common intermediate $\Delta\tilde{\mu}H$, 2 such models and a leak, they were able to analyze in detail how non-linear relationships between the flows and $\Delta\tilde{\mu}H$ modify the results of fig.1, and more generally the results of double inhibitor titrations [10]. An interesting conclusion of this study is that, depending on the kinetics, a given concentration of uncoupler can be equally, less, or more effective in inhibiting the rate of ATP synthesis in the presence of an ATPase inhibitor as compared with its absence. In conclusion, the uncoupler-inhibitor titration approach by itself cannot unequivocally discriminate between delocalized chemiosmotic mechanisms and other mechanisms. On the other hand, it will be shown in [10] that the approach can so discriminate if accompanied by appropriate additional measurements.

REFERENCES

[1] O'Shea, P.S. and Thelen, M. (1984) FEBS Lett. 176, 79–82.

[2] Van der Bend, R.L. and Herweijer, M.A. (1985) FEBS Lett. 186, 8–10.

[3] Hitchens, G.D. and Kell, D.B. (1983) Biochim. Biophys. Acta 723, 308–316.

[4] Westerhoff, H.V., Melandri, B.A., Venturoli, G., Azzone, G.F. and Kell, D.B. (1984) Biochim. Biophys. Acta 768, 257–292.

[5] Ferguson, S.J. (1985) Biochim. Biophys. Acta 811, 47–97.

[6] Berden, J.A., Herweijer, M.A. and Cornelissen, J.B.W.J. (1984) in: H^+-ATPase (ATP Synthase) Structure, Function, Biogenesis. The F_0-F_1 Complex of Coupling Membranes (Papa, S. et al. eds) pp.339–348, ICSU Press and Adriatica Editrice, Bari.

[7] Zoratti, M., Pietrobon, D. and Azzone, G.F. (1982) Eur. J. Biochem. 126, 443–451.

[8] Van Dam, K., Westerhoff, H.V., Krab, K., Van der Meer, R. and Arents, J.C. (1980) Biochim. Biophys. Acta 591, 240–250.

[9] Baum, H., Hall, G.S., Nalder, J. and Beechey, R. (1971) in: Energy Transduction in Respiration and Photosynthesis, pp.747–755, Adriatica Editrice, Bari.

[10] Pietrobon, D. and Caplan, S.R. (1985) submitted.

[11] Cotton, N.P.J. and Jackson, J.B. (1983) FEBS Lett. 161, 93–99.

[12] Mills, J.D. (1984) in: H^+-ATPase (ATP synthase) Structure, Function, Biogenesis. The F_0-F_1 Complex of Coupling Membranes (Papa, S. et al. eds) pp.349–358, ICSU Press and Adriatica Editrice, Bari.

[13] Pietrobon, D. and Caplan, S.R. (1985) Biochemistry, in press.