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Uncoupler-inhibitor titrations of ATP-driven reverse electron transfer in isolated rat-liver mitochondria

J. Sánchez Olavarría*, A. Lambers and K. van Dam

Laboratory of Biochemistry, University of Amsterdam, Amsterdam (The Netherlands)

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Uncoupler-inhibitor titrations of ATP-driven reverse electron transfer across the first site of the respiratory chain were performed in isolated rat-liver mitochondria, and the experimental results were compared with the predictions of a simple delocalised chemiosmotic mechanism. The rates of ATP hydrolysis (J_p) and reverse electron transfer ($-J_0$) were measured at different uncoupler (S-13) concentrations, either in the absence or in the presence of rotenone. When the rates $-J_0$ and J_p measured at different uncoupler concentrations were expressed as percentages of the activity at zero uncoupler concentration, it was found that the efficiency of S-13 to uncouple the reverse electron transfer and to stimulate ATP hydrolysis was not significantly changed upon partial inhibition with rotenone. These results are in contrast with data from a study of uncoupler-inhibitor titrations in submitochondrial particles published previously, in which a higher effectiveness of several uncouplers to inhibit ATP-driven reverse electron transfer was observed in the presence of rotenone.

Introduction

The role of chemiosmotic proton circuits in energy transduction in biological membranes is widely accepted, although the detailed mechanism of the process is still a matter of debate. According to Mitchell's chemiosmotic hypothesis [1], a proton electrochemical gradient ($\Delta\tilde{\mu}_{H^+}$) is the sole and competent intermediate between the respiratory chain (the input or primary proton pump in the chemiosmotic circuit) and the ATP synthase (the output or secondary proton pump in the chemiosmotic circuit). Nevertheless, during recent years considerable experimental evidence has been reported that seems to disagree with this delocalised coupling mechanism and, therefore, alternative hypotheses have been presented in which the proton circuits are confined to small coupling units of the membrane without full equilibration with the bulk aqueous phases at both sides of the membrane [2,3].

Double-inhibitor titrations and uncoupler-inhibitor titrations have been used as means to discriminate between the localised or delocalised natures of the mechanism of coupling in photophosphorylation or oxidative phosphorylation, the advantage of this approach being its independence of accurate measurements of $\Delta\tilde{\mu}_{H^+}$ [4–6]. Double-inhibitor or uncoupler-inhibitor titrations were considered to form a clear-cut experimental

* Present address: Department of Biochemistry and Molecular Biology, University of Alcalá de Henares, Alcalá de Henares, Madrid, Spain.

Abbreviations: Mops, 4-morpholinepropanesulphonic acid; MNET, mosaic non-equilibrium dynamics.

Correspondence: K. van Dam, Laboratory of Biochemistry, University of Amsterdam, P.O. Box 20151, 1000 HD Amsterdam, The Netherlands.

approach that could discriminate without ambiguity between the localised and the delocalised types of coupling mechanism. In the case of uncoupler-inhibitor titrations, it was expected that, depending on the localised or delocalised character of the proton circuit, the relative decrease in the output flow (energy-driven reaction) by a given uncoupler concentration should increase or decrease, respectively, in the presence of an inhibitor of the output proton pump. The observation that the effectiveness of an uncoupler increased upon partial inhibition of the output pump has been taken as strong evidence against a delocalised coupling mechanism [5–7].

The validity of this interpretation has been questioned recently when these experiments were analysed in a more quantitative way [8–14]. For instance, it was possible to simulate the 'anomalous' results of uncoupler-inhibitor titrations with a delocalised model of energy coupling that assumes linear relationships between the flows and the forces [8,9]. The same authors also showed that previous results of titrations, formerly considered as inconsistent with a delocalised coupling mechanism, can be simulated with a delocalised model that assumes non-linear flow-force relationships, provided that certain kinetic requirements are satisfied [10]. Evidently, better knowledge of the characteristics of the system (kinetic constants and stoichiometries of the proton pumps, magnitude of the leak, etc.) is needed for a correct interpretation of the results of double-inhibitor or uncoupler-inhibitor titrations. In the absence of such detailed information, the question arises of whether or not the shape of the experimental titration curves can be simulated when actual or measured values of the parameters (rather than arbitrarily chosen ones) are used in the computations.

In bovine heart submitochondrial particles, Herweijer et al. [7] observed a higher effectiveness of several uncouplers in inhibiting the ATP-driven reverse electron transfer in the presence of rotenone than in its absence. Using realistic values (derived from actual experiments) for the different parameters it was not possible to simulate the observed titration curves with a simple delocalised model of coupling and linear flow-force relationships. Although the possibility of simulating the results with non-linear flow-force relationships still remains, the different behaviour of uncouplers of the carrier type (their effectiveness was increased upon inhibition with rotenone) and the pore-forming uncoupler gramicidin (its effectiveness was not increased in particles inhibited with rotenone) is difficult to explain with a delocalised coupling mechanism. A difficulty with these experiments in submitochondrial particles, however, is the fact that the major part of ATP hydrolysis is not coupled, presumably as a consequence of a high permeability of the membranes to protons. In this paper we report uncoupler-inhibitor titrations of ATP-driven reverse electron transfer in mitochondria, that have a much lower proton permeability than submitochondrial particles. It is shown that the effectiveness of the protonophorous uncoupler S-13 to inhibit the reverse electron transfer in intact mitochondria did not change upon inhibition with rotenone, in contrast with the results obtained with submitochondrial particles. We also confirmed and extended the analysis of uncoupler-inhibitor titrations with a linear model of delocalised coupling as reported by Pietrobon and Caplan [8] and show that, although this model predicts a higher effectiveness of an uncoupler when the output pump is partially inhibited, this effect may not be noticed in actual experiments.

Materials and Methods

Rat-liver mitochondria were isolated as described previously [15]. The protein content was measured by the biuret method using bovine serum albumin as a standard. Mitochondria (2 mg/ml) were incubated in an Oxygraph vessel, with continuous stirring, in a medium containing 10 mM Mops, 2 mM EGTA, 2 mM succinate, 2 mM acetoacetate, 0.2 mM malate, 0.122 mM D,L-3-hydroxybutyrate, 0.2 mM ADP and 240 mM mannitol (pH 7.15). Antimycin was added to give a final concentration of 0.2 μg/mg protein; 2 mM cyanide was also present in the incubation medium. Rotenone and S-13 were used at the concentrations indicated in the figures. All the incubations were carried out at 25°C under argon atmosphere. Incubations were started by the addition of 5 mM ATP. Samples (0.5 ml) were withdrawn immediately after the additon of ATP

($t = 0$ min) and at 2-min intervals during a 10-min period. Samples were deproteinised with an equal volume of a mixture of phenol/chloroform/isoamyl alcohol (38 : 24 : 1, v/v) and mixed thoroughly for 1 min. After centrifuging at 13000 rpm for 2 min in an Eppendorf centrifuge, the aqueous layer was extracted twice with chloroform and *n*-hexane. Phosphate was analysed according to the method of Chen et al. [16] and 3-hydroxybutyrate was analysed using the method of Williamson et al. [17]. ATP hydrolysis and reverse electron transfer were calculated from respectively, the plots of phosphate and 3-hydroxybutyrate production against incubation time.

3-Hydroxybutyrate, acetoacetate, succinate, L-malate, EGTA and Mops were from Sigma (St. Louis, U.S.A.), whereas ADP, ATP, malate dehydrogenase and 3-hydroxybutyrate dehydrogenase were from Boehringer (Mannheim, F.R.G.). S-13 was a gift from Dr. P. Heytler.

Results and Discussion

The rates of ATP hydrolysis (J_p) and reverse electron transfer ($-J_0$) from succinate to acetoacetate in isolated rat-liver mitochondria were titrated with S-13 either in the absence or in the presence of rotenone (3 and 6 ng/mg protein). The results shown in Fig. 1 indicate that 3-hydroxybutyrate production was more sensitive to rotenone inhibition than ATP hydrolysis in the absence of uncoupler. Addition of S-13 produced a decrease in reverse electron transfer and an enhancement of ATP hydrolysis. The absolute decrease in the redox reaction was higher at lower rotenone concentrations, whereas the increase in the rate of ATP hydrolysis was approximately the same for all rotenone concentrations used.

In Fig. 2 the relative rates of ATP hydrolysis and reverse electron transfer have been plotted against the uncoupler concentrations at different rotenone concentrations. These results indicate that there is hardly any effect of rotenone on the effectiveness of S-13 to inhibit reverse electron transfer from succinate to acetoacetate or to stimulate ATP hydrolysis.

It has recently been pointed out that the validity of double-inhibitor or uncoupler-inhibitor titrations to discriminate between delocalised and

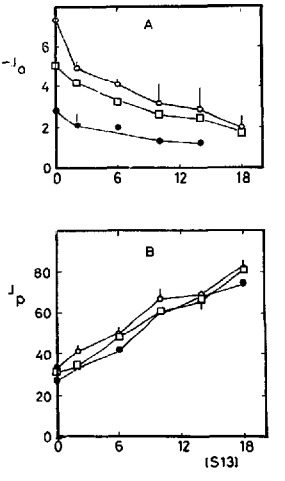

Fig. 1. S-13 and rotenone titrations of ATP-driven reverse electron transfer in rat-liver mitochondria; ○———○, no rotenone was present; □———□, 3 ng rotenone/mg protein; ●———●, 6 ng rotenone/mg protein. Reverse electron transfer (panel A; nmol/min per mg protein) and ATP hydrolysis rate (panel B; nmol/min per mg protein) were measured from 3-hydroxybutyrate and phosphate production along the incubation period. Error bars are given for some representative points; they indicate S.E. (in one direction only) for at least three independent experiments.

localised coupling mechanisms in oxidative phosphorylation should not be overestimated [8,9]. Nevertheless, experiments in which the activity of an energy-dependent process is titrated with an inhibitor of the primary proton pump or with an uncoupler, either in the presence or in the absence of an inhibitor of the secondary proton pump, can still offer some interesting clues as to the mechanism of energy transduction. The usual approach is to compare the experimental results of such titrations with the predictions derived from various coupling mechanisms that rely on different assumptions about the nature of the high-energy intermediate. In this aspect, mosaic non-equilibrium thermodynamics (MNET) provides a

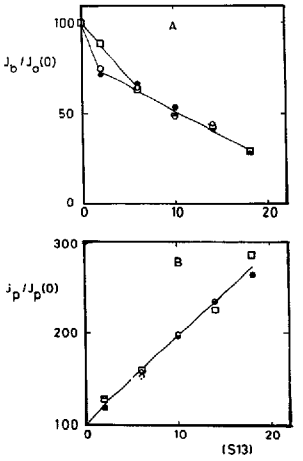

Fig. 2. Normalised rates of reverse electron transfer and ATP hydrolysis at different S-13 concentrations in rat-liver mitochondria, partially inhibited with rotenone. Data from Fig. 1 are replotted as percentages of the activity measured in the absence of S-13 at a given rotenone concentration: reverse electron transfer (panel A) and ATP hydrolysis (panel B).

means to formulate a quantitative description of the biological energy-transducing system [14]. If we assume certain relationships between the flows (rates) and the forces, it is possible to derive equations relating the rate of the energy-driven process to parameters, the values of which can be experimentally modified or are supposed to be constant. In the case that the predictions of a given model do not fit the experimental results, the model is modified (usually it becomes more and more complicated) by the introduction of new parameters, changes in the postulated flow-force relationships, etc. and it is tested again.

For uncoupler-inhibitor titrations it has been demonstrated the the calculated inhibition of the energy-dependent process by an uncoupler can be either higher or lower in the presence of an inhibitor of the secondary proton pump than in its absence in a delocalised coupling mechanism, depending on the non-linear nature of the flow-force relationships and certain kinetic constraints [9]. Uncoupler-inhibitor titrations of ATP-driven reverse electron transfer in bovine heart submitochondrial particles resulted in a sensitivity to inhibition by lipophilic uncouplers, but not to inhibition by gramicidin, that was higher in the presence of rotenone than in its absence [7]. These results seem difficult to accommodate in a delocalised coupling mechanism, irrespective of the assumed flow-force relationships, unless different kinds of uncoupler affect the secondary proton pump in very specific ways.

We carried out similar uncoupler-inhibitor titrations in intact rat-liver mitochondria and analysed them with a simple delocalised model that assumes proportional relationships between the flows and the thermodynamic forces that drive them (see the Appendix for a description of the model and the derivation of relevant equations). From this model it follows that the initial slopes of the titration curves (relative rate versus uncoupler concentration) as well as the flux control of the leak pathway should increase (i.e., become more negative) in the presence of an inhibitor of the secondary proton pump. In apparent contradiction with what is expected from the model, our results indicate that there is hardly any effect of rotenone on the effectiveness of S-13 to inhibit the redox proton pump. In order to ascertain the extent to which the prediction of the model and the experimental results are not in agreement, it is necessary to compute the appropriate function (initial slope or flux control) at different rotenone concentrations, using measured or calculated values of the parameters in the equations (L_0, L_p, n_0, n_p).

According to this simple delocalised model, a linear relationship between the reversal rate and the ATP-hydrolysis rate should be observed when the passive flow of H^+ across the inner membrane is titrated with an uncoupler. In Fig. 3, it is shown that the measured reversal rate and ATP-hydrolysis rate are in fact linearly related (data taken from Fig. 1). Moreover, for different rotenone concentrations it is predicted, and within experimental error observed, that the lines intersect on the X-axis. This plot can be used to calculate the values of the coefficients L_p and L_0 under our

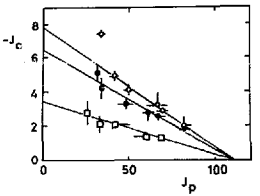

Fig. 3. Relation between rates of reverse electron transfer and ATP hydrolysis at different S-13 concentrations in rat-liver mitochondria, in the presence of 0 (○———○), 3 (●———●) and 6 (□———□) ng rotenone per mg protein. Data from Fig. 1 are replotted.

experimental conditions, if we assume certain values for n_0 and n_p. Although the proton stoichiometry of Site 1 and ATPase are being discussed, we have assumed that $n_0 = n_p = 3$. The values of L_p and L_0 at different rotenone concentrations can now be used for the computation of the value of the flux control coefficient at different values of L_0. It was found that the value of this control coefficient of uncoupler was essentially constant over a wide range of L_0 values that include the calculated L_0 values in the actual experiments. Hence, although the model predicts a higher sensitivity of the reversal rate to S-13 in the presence of rotenone than in its absence, it turns out that this effect is very small when realistic values of the parameters are used for the calculations. We suggest, therefore, that no further refinements of the model are necessary to accommodate the experimental results of uncoupler-inhibitor titrations in isolated intact mitochondria. This conclusion is in contrast with that for submitochondrial particles, where a direct coupling mechanism was postulated [7]. The reason for the different behaviour of intact mitochondria and submitochondrial particles in uncoupler-inhibitor titrations still remains unclear.

Appendix

MNET model for the ATP-driven reverse electron transfer

A simple linear model of delocalised coupling based on mosaic non-equilibrium thermodynamics (MNET) as described by Van Dam et al. [15,18] has been used for the analysis of the experimental results. From the steady-state condition of zero net flow of protons across the membrane, it is possible to derive the equations for the rate of ATP hydrolysis (J_p) and reverse electron transfer ($-J_0$):

$$J_p = \frac{L_p\left[\left(L_H + L_0 n_0^2\right)\Delta G_p - L_0 n_0 n_p \Delta G_0\right]}{L_H + L_0 n_0^2 + L_p n_p^2} \tag{1}$$

$$-J_0 = \frac{L_0\left[L_p n_0 n_p \Delta G_p - \left(L_H + L_p n_p^2\right)\Delta G_0\right]}{L_H + L_0 n_0^2 + L_p n_p^2} \tag{2}$$

where ΔG_p and ΔG_0 are the free energy changes of the ATP hydrolysis and the redox reaction (malate + 3-hydroxybutyrate → succinate + acetoacetate), respectively; n_p and n_0 stand for the number of protons that are translocated per ATP molecule hydrolysed and per two electrons transferred across the first site, respectively. L_H is the proportionality constant between the passive flow of protons across the membrane and the force $\Delta\tilde{\mu}_{H^+}$. L_p and L_0 are the proportionality constants between each flow (J_p or $-J_0$) and the respective total thermodynamic force ($\Delta G_p + n_p \Delta\tilde{\mu}_{H^+}$; $\Delta G_0 + n_0 \Delta\tilde{\mu}_{H^+}$). These equations hold as long as the forces ΔG_p and ΔG_0 are kept constant.

Eqns. 1 and 2 describe how J_p and $-J_0$ change when the leak is modified by adding increasing ammounts of uncoupler (L_H increases) either in the absence or in the presence of rotenone (L_0 is larger in the first situation) at constant values of the other parameters. Another useful equation can be derived by eliminating L_H from Eqns. 1 and 2:

$$-J_0 = \frac{-L_0 n_0}{L_p n_p} J_p + \frac{L_0}{n_p}\left(n_0 \Delta G_p - n_p \Delta G_0\right) \tag{3}$$

This equation relates the changes of the flows J_p and $-J_0$ when the passive flow of protons through the membrane is titrated with an uncoupler.

Uncoupler-inhibitor titration

In order to express the effectiveness of an uncoupler to inhibit the output flow in the presence of a specific inhibitor of the output proton pump, several parameters can be considered, for instance

(i) the titre required for full or half uncoupling of the output pump, or (ii) the initial slope of the titration curve (relative output flow versus uncoupler concentration). As already discussed by Pietrobon and Caplan [8], the linear model predicts that the uncoupler titre required for full inhibition of the output pump is not changed upon partial inhibition of the output pump. Here we show that according to the same model, the initial slope of the titration curve is higher in the presence of the inhibitor of the output pump.

If we take the approximation that L_H is either zero or much smaller than $L_p n_p^2$ in the absence of added uncoupler, Eqn. 2 can be simplified to:

$$-J_0(0) = \frac{L_0 L_p n_p (n_0 \Delta G_p - n_p \Delta G_0)}{L_0 n_0^2 + L_p n_p^2} \quad (4)$$

Then, the relative rate of reverse electron transfer at any uncoupler concentration is the ratio $-J_0$ (Eqn. 2) over $-J_0(0)$ (Eqn. 4):

$$\frac{-J_0}{-J_0(0)} = \frac{\left[L_p n_0 n_p \Delta G_p - \left(L_H + L_p n_p^2\right)\Delta G_0\right]\left(L_0 n_0^2 + L_p n_p^2\right)}{L_p n_p \left(n_0 \Delta G_p - n_p \Delta G_0\right)\left(L_H + L_0 n_0^2 + L_p n_p^2\right)} \quad (5)$$

After taking the derivative and substituting $L_H = 0$:

$$\left(\frac{\mathrm{d}\left(\frac{-J_0}{-J_0(0)}\right)}{\mathrm{d}\, L_H}\right)_{L_H = 0} = \frac{-n_0\left(L_0 n_0 \Delta G_0 + L_p n_p \Delta G_p\right)}{L_p n_p \left(n_0 \Delta G_p - n_p \Delta G_0\right)\left(L_0 n_0^2 + L_p n_p^2\right)} \quad (6)$$

This equation shows how the initial slope of the titration curve depends on L_0 (which is inversely related to the rotenone concentration) at constant values of the other parameters.

An alternative approach to estimate how the contribution of the leak pathway to the control of the reverse electron transfer changes in the presence of rotenone is to calculate the flux control of the leak at different L_0 values. From the definition

$$J_{0}C_{1} = \frac{\mathrm{d}(-J_0)}{\mathrm{d}\, L_H} \frac{L_H}{(-J_0)}$$

and using Eqn. 2 it follows that:

$$J_{0}C_{1} = \frac{-L_H n_0 \left(L_0 n_0 \Delta G_0 + L_p n_p \Delta G_p\right)}{\left[L_p n_0 n_p \Delta G_p - \left(L_H + L_p n_p^2\right)\Delta G_0\right]\left(L_H + L_0 n_0^2 + L_p n_p^2\right)} \quad (7)$$

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