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Analysis of mechanisms of free-energy coupling and uncoupling by inhibitor titrations: theory, computer modeling and experiments

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The rates of ATP synthesis and of ATP-driven NAD reduction have been measured in bovine heart submitochondrial particles as a function of the fraction of inhibited redox pumps (in titrations with either antimycin or rotenone) and of the fraction of inhibited ATPases (in titrations with DCCD). The flux control coefficients of the redox and ATPase proton pumps on the rates of ATP synthesis and of ATP-driven NAD reduction have been derived and found to be equal to 1 for both pumps; i.e., both pumps appear to be 'completely rate limiting'. A theoretical analysis of the inhibitor titration approach based on kinetic models of chemiosmotic coupling and on the theory of metabolic control is presented. This analysis (i) shows that the results of the single inhibitor titrations are incompatible with a delocalized chemiosmotic mechanism of energy coupling if the proton conductance of the membrane is sufficiently low with respect to the conductances of the pumps; and (ii) suggests an experimental approach based on the determination of the P/O and the respiratory control ratios at different degrees of inhibition of the proton pumps to establish the origin of the 'loose coupling' of submitochondrial particle preparations. Three independent types of observation show that the 'loose coupling' of the particle preparation is not mainly due to an increased membrane proton conductance. The same and other independent observations are consistent with the view that the loose coupling of submitochondrial particle preparation is due mainly to inhomogeneity, i.e. to the presence of a subpopulation of highly leaky non-phosphorylating vesicles respiring at maximal rate. The results as a whole together with the simulations and analysis presented lead to the conclusion that the mechanism of free-energy coupling in submitochondrial particles is not completely delocalized.

Introduction

Membrane-linked biological free-energy transduction is accomplished by primary ($\Delta\tilde{\mu}_H$ gener-

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Abbreviations: $\Delta\tilde{\mu}_H$, transmembrane electrochemical proton gradient; ΔpH, transmembrane pH gradient; $\Delta\psi$, transmembrane electrical potential gradient; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; DCCD, *N*,*N*′-dicyclohexylcarbodiimide; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; J_e, rate of electron transfer; RCR, respiratory control ratio; SMP, submitochondrial particle; Ap_5A, P^1P^5-di(adenosine-5′-)pentaphosphate.

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ating) redox or light driven proton pumps and secondary ($\Delta\tilde{\mu}_H$ utilizing) ATPsynthetase proton pumps. The question of whether the proton pathway between the two types of pump is delocalized within the two bulk aqueous phases on each side of the membrane (delocalized chemiosmotic coupling theory) [1] or is (at least in part) localized within the membrane or at its surface remains open (for reviews see Refs. 2 and 3). Among the different approaches used to discriminate between delocalized and localized mechanisms of energy coupling the double inhibitor titrations described originally by Baum and coworkers [4] and more recently by Kell and coworkers [5–7] has become very popular. The reason has been mainly its supposed independence from the measurement of $\Delta\tilde{\mu}_H$.

However, Pietrobon and Caplan [8,9] have shown recently that the results predicted by a delocalized chemiosmotic model in this type of experiment are strictly dependent on (i) the kinetics of the pumps, i.e., on the type of relations existing between rate of ATP synthesis and rate of electron transfer and $\Delta\tilde{\mu}_H$ and on (ii) the size and the kinetics of the leak. In order to draw an unequivocal conclusion about the localization/delocalization of the system from a double inhibitor titration it is then necessary to measure the relations between flows and $\Delta\tilde{\mu}_H$ for the two proton pumps and for the leak. The approach thus looses its main claimed advantage (the independence from the measurement of $\Delta\tilde{\mu}_H$) as means of discrimination between different energy-coupling mechanisms.

In this paper we use inhibitor titrations of the primary and secondary proton pumps in a different way to establish the extent of delocalization of energy coupling in submitochondrial particles. Instead of comparing the relative inhibition of the rate of ATP synthesis by, e.g., an inhibitor of the redox pumps in the presence and absence of a given amount of inhibitor of the ATPases (double-inhibitor titrations) we compare the relative inhibition of the rate of ATP synthesis by low amounts of an inhibitor of the redox pumps and by low amounts of an inhibitor of the ATPases (single-inhibitor titrations). We introduce the name 'single inhibitor titrations' (even if we are actually using two inhibitors) to distinguish this approach from the double inhibitor titrations approach introduced by Kell and coworkers. A theoretical analysis of the single-inhibitor approach based on kinetic models of chemiosmotic coupling with linear and non-linear relations between flows and forces and also on the theory of metabolic control [10–12] is presented. The analysis shows that some results of the single-inhibitor titrations do allow an unequivocal discrimination between delocalized and localized energy-coupling mechanisms without the need of measuring $\Delta\tilde{\mu}_H$. For a correct interpretation of the results it is, however, necessary to have a measure of the dissipative proton flow (leak) and to establish the cause of the well-known 'loose coupling' of the submitochondrial particle preparations. The modelling study suggests an experimental approach based on the determination of the P/O ratio and the RCR at different degrees of inhibition of the proton pumps to get these essential informations, again without the need of measuring $\Delta\tilde{\mu}_H$. The results as a whole together with the simulations and analysis presented lead to the conclusion that the mechanism of free-energy coupling in SMP's is not (or is not completely) delocalized.

Experimental

Mitochondrial preparations. Bovine heart mitochondria were prepared according to the procedure of Azzone et al. [13], except that 2 M Tris instead of triethanolamine was used to adjust the pH. The upper layer of the mitochondrial sediment was discarded and the lower dark layer was suspended in a medium containing 0.25 M sucrose, 10 mM Tris/Cl and 1 mM succinate/Tris; the suspension was recentrifuged and the pellet suspended in a small volume of the same medium to give a final concentration of about 70 mg protein per ml. Aliquots of 5 ml of mitochondrial suspension were stored at $-20\,^{\circ}C$.

To prepare bovine heart submitochondrial particles [44], 5 ml of the above-mentioned mitochondrial preparation were thawed and suspended in 15 ml medium at a final concentration of 0.25 M sucrose, 10 mM Tris/Mops, 5 mM ATP, 5 mM $MgCl_2$, 10 mM $MnCl_2$, 5 mM succinate and 2 mM dithiothreitol (pH 7.5). After saturation with nitrogen, the suspension was ex-

posed, eight times for periods of 15 s with 30-s intervals, to sonic oscillation in a Branson B30 sonifier at 120 W power. Then the suspension was centrifuged at 13500 r.p.m. (SS 34 Sorvall rotor) for 10 min and, after incubation in the presence of 5 mM malonate at room temperature for 30 min in order to activate the succinate dehydrogenase [40], the supernatant was centrifuged in a Beckman ultracentrifuge at 45000 r.p.m. (50 TI rotor) for 40 min. The sediment of particles was suspended in a medium containing 0.25 M sucrose, 10 mM Tris/Mops and 1 mM succinate/Tris (pH 7.5) to a final concentration of about 50 mg per ml protein. Aliquots of 1 ml of submitochondrial suspension were stored at −20 °C and utilized within 1 week from the sonication.

Determination of the rate of ATP-driven NAD reduction. Submitochondrial particles were suspended (0.1–0.2 mg per ml) in the medium described in the legend to Fig. 6 in thermostatically controlled cuvettes. The medium contained a non-limiting amount of creatine kinase and phosphocreatine to regenerate ATP, and antimycin to avoid electron transfer to oxygen. After 8 min of incubation in State 4 in the presence of variable inhibitor (either DCCD or rotenone) concentrations, 0.5 mM NAD was added and NADH formation was monitored spectrophotometrically at 340 nm ($\varepsilon = 6.2\ mM^{-1} \cdot cm^{-1}$). With DCCD as inhibitor the particles were preincubated for 40 min in the presence of the desired inhibitor concentration.

Determination of the rate of ATP synthesis. Submitochondrial particles were incubated (0.2–0.3 mg per ml) in the presence of the respiratory substrate (succinate or durohydroquinone) and variable concentrations of inhibitors (either DCCD or antimycin) in the appropriate medium. In the durohydroquinone-driven ATP synthesis, durohydroquinone was generated by reduction of duroquinone (50 μM) with non-limiting amount of NADH and diaphorase [14] (see legend to Fig. 8). After 8 min of incubation in State 4, in the presence of non-limiting amount of hexokinase and glucose, 0.5 mM ADP was added. The rate of ATP synthesis was assayed by a sampling procedure as described by Zoratti and Petronilli [15].

Determination of the rate of ATP hydrolysis. Submitochondrial particles were suspended in the appropriate medium for the same time and in the same conditions as in the parallel determinations of the rate of ATP synthesis or ATP-driven NAD reduction. A fully uncoupling amount of FCCP (1.5 μmol per mg) was present to obtain maximal stimulation of the rate of ATP hydrolysis. The medium contained non-limiting amounts of phospho*enol*pyruvate and pyruvatekinase/lactate dehydrogenase to couple the ATP hydrolysis to the NADH oxidation which was monitored spectrophotometrically at 340 nm ($\varepsilon = 6.2\ mM^{-1} \cdot cm^{-1}$). ATP hydrolysis was started by the addition of ATP. The rate of ATP hydrolysis was corrected for the rate of ATP hydrolysis measured in the presence of excess DCCD.

Determination of the respiratory rate. Rates of respiration were determined from the rate of oxygen consumption by submitochondrial particles incubated in a closed, thermostatted and stirred vessel, measured with a Clark electrode (Yellow Springs, OH). Determination of medium oxygen content and calibration of the electrode response were carried out by allowing submitochondrial particles to oxidize known (spectrophotometrically determined) amounts of NADH. The rates of respiration with durohydroquinone as substrate were corrected for the oxygen uptake in the presence of completely inhibitory concentrations of KCN.

Determination of passive proton efflux. Submitochondrial particles (0.5–1 mg per ml) were suspended in a medium containing 0.25 M sucrose, 2 mM EGTA, 4 μM rotenone, 20 mM succinate, 30 mM KCl, 5 μg per mg oligomycin, 0.5 μg per mg valinomycin. After 3 min of incubation in State 4, 2 μg antimycin per mg protein were added. The passive proton efflux was measured by monitoring medium pH changes following inhibition of respiration. Rates of H^+ efflux were estimated from the initial, practically rectilinear part of the recording after antimycin addition and quantitated by double titration with standard solutions of NaOH and HCl. Doubling the amount of protein led to doubling of the rate of proton efflux.

Materials. Protein concentrations were determined by the biuret method using bovine serum albumin as standard. Enzymes, nucleotides, inhibitors were purchased from Sigma. Spectropho-

tometric measurements were carried out using a Perkin-Elmer Lambda 5 spectrophotometer equipped with temperature control. All measurements were carried out under thermostatically controlled conditions at 25 °C (pH 7.3).

Theory

Pietrobon and Caplan [8] have shown that for the simple chemiosmotic protonic circuit constituted by a redox (primary or $\Delta\tilde{\mu}_H$ generating) proton pump and an ATPsynthase (secondary or $\Delta\tilde{\mu}_H$ consuming) proton pump, with linear relations between flows and $\Delta\tilde{\mu}_H$, the relative inhibition of the rate of ATP synthesis by a given concentration of an inhibitor of either pump is exclusively dependent on the ratio r between the conductances of the two pumps in the circuit. The conductance of the redox pumps represents the 'readiness' with which the proton flow of the redox pumps, $J_{H,e}$, changes in response to a change in $\Delta\tilde{\mu}_H$, and is given, in the linear model just mentioned, by the slope, L_e, of the plot $J_{H,e}$ vs. $\Delta\tilde{\mu}_H$ at a given affinity (instantaneous negative Gibbs free energy) of the redox reaction. The conductance of the ATPsynthases is given by the slope L_p of the plot $J_{H,p}$ vs $\Delta\tilde{\mu}_H$ at a given affinity of the ATP hydrolysis reaction.

The lower the conductance of the ATP synthases with respect to that of the redox pumps, the lower is the relative inhibition of the rate of ATP synthesis for a given fraction of inhibited redox pumps and vice versa, the higher is the relative inhibition of the rate of ATP synthesis for a given fraction of inhibited ATP synthases. This result is illustrated for a redox inhibitor in Fig. 1, which shows the simulated relative inhibition of the rate of ATP synthesis for different values of $r = L_p/L_e$ as a function of the inhibition factor, f_e (varying from 1 at zero inhibitor concentration to 0 at the concentration of inhibitor that inhibits all the pumps). The dashed lines refer to the two limiting cases: $r \to \infty$ for which $J_p/J_p(0) = f_e$, i.e., the inhibition of the rate of ATP synthesis is proportional to the fraction of inhibited redox pumps, and $r \to 0$ for which $J_p/J_p(0) = 1$, i.e., the rate of ATP synthesis is insensitive to inhibition of the redox pumps. The simulated titration curves obtained with an inhibitor of the ATP synthases are

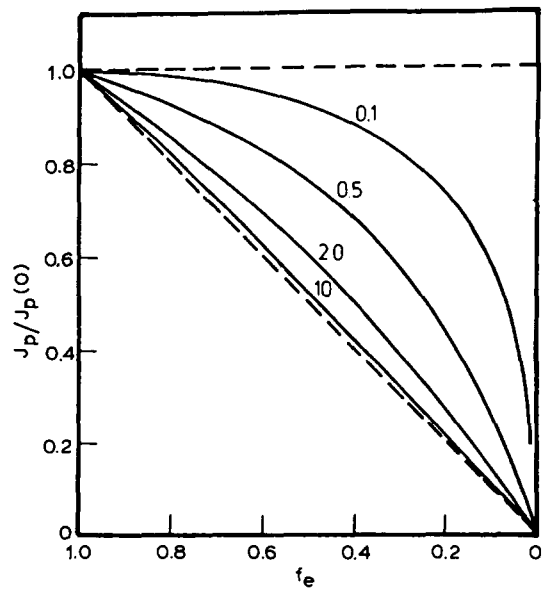

Fig. 1. Simulations of the relative rate of ATP synthesis, $J_p/J_p(0)$, as a function of the inhibition factor of the redox pumps, f_e. The simulations are obtained on the basis of the chemiosmotic protonic circuit constituted by a redox proton pump and an ATP synthase proton pump assuming linear relations between flows and $\Delta\tilde{\mu}_H$. $J_p(0)$ is J_p at $f_e = 1$. The different curves refer to different values of the ratio between the conductances of the two proton pumps, $L_p/L_e = r$, which are indicated in the figure. The computations are based on the equation: $J_p/J_p(0) = (1 + r)/(1 + r/f_e)$ [8]. See text.

inversely symmetrical to those obtained with a redox inhibitor, i.e., for, let's say, $r = 0.1$ the titration curve with an ATP synthase inhibitor is identical to the titration curve corresponding to $r = 10$ in Fig. 1.

It is interesting to relate these results to the metabolic control theory developed by Kacser and Burns [10] and Heinrich and Rapoport [11], and recently extended to the analysis of free-energy transduction by Westerhoff and colleagues [12,16–18]. In this theory the control exerted by the enzyme i in a given metabolic pathway is quantified in terms of the flux-control coefficient, C_i^v, defined as the relative effect of a change in concentration of enzyme i, $[e_i]$, on the steady-state rate, v:

$$C_i^v = \frac{\partial v[e_i]}{v\partial[e_i]} \qquad (1)$$

Since the total control on a flux must be unity,

$$\Sigma_i C_i^v = 1 \qquad (2)$$

This is the so called flux-control summation theorem.

In the simple chemiosmotic system considered above the control on the steady-state rate of phosphorylation is distributed between the redox and ATP synthase pumps. It can be shown that the flux-control coefficients of the pumps are uniquely dependent on the ratio r ($= L_p/L_e$) between their conductances [8,17–19]. We have just seen that the relative inhibition of the rate of phosphorylation by a given concentration of inhibitor of either pump is uniquely dependent on the same ratio r. Actually, remembering that f_e corresponds to the fraction of active (not inhibited) redox pumps the flux-control coefficient of the redox pumps can be derived graphically from the titration curves of Fig. 1 [20,21]. In fact, this coefficient is given (cf. Eqn. 1), for each r, by the tangent to the corresponding titration curve at $f_e = 1$. As r decreases the flux control of the redox pumps decreases going from 1 when $r \to \infty$ (redox pumps completely rate limiting) to 0 when $r \to 0$ (ATP synthases completely rate limiting). The flux control of the ATP synthases is given by the tangent at $f_p = 1$ to the titration curves obtained with an ATP synthase inhibitor. These titration curves are inversely symmetrical to those of Fig. 1, since the sum of the flux-control coefficients of the two pumps is always equal to 1 (cf. Eqn. 2).

The above discussion holds if a passive pathway of proton diffusion across the membrane is not included in the chemiosmotic system or, if included, when the leak membrane conductance, L_H^l, has a negligible value with respect to the conductances of the pumps [8,17,18]. Fig. 2 shows the titration curves corresponding to those of Fig. 1 when a non-negligible parallel ohmic leak is included in the linear chemiosmotic circuit. The titration curves with a redox and an ATP synthase inhibitor are no longer inversely symmetrical, since now in the flux-control summation theorem (Eqn. 2) applied to the rate of ATP synthesis the flux-control coefficient of the leak must also be considered. The flux-control coefficient of the leak is negative, since an increase in the leak conductance

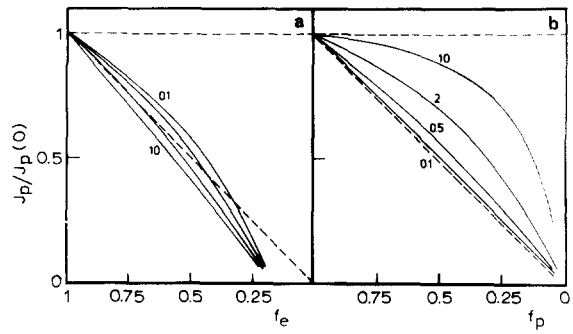

Fig. 2. Simulations of the relative rate of ATP synthesis, $J_p/J_p(0)$ as a function of the inhibition factor of the redox pumps, f_e (a) and of the inhibition factor of the ATPase pumps, f_p (b). The simulations are obtained on the basis of the protonic chemiosmotic circuit constituted by a redox proton pump, an ATP synthase proton pump and a leak assuming linear relations between flows and $\Delta\tilde{\mu}_H$. The different curves refer to different values of r ($= L_p/L_e$) which are indicated in the figure. In each case $L_H^l = 0.5\ L_e$. Computations in panel a are based on the equation:

$$\frac{J_p}{J_p(0)} = \frac{\left(1 + r + \frac{L_H^l}{L_e}\right)\left(1 - \frac{L_H^l}{f_e L_e\left(\frac{n_p A_e}{n_e A_p} - 1\right)}\right)}{\left(1 + \frac{r}{f_e} + \frac{L_H^l}{f_e L_e}\right)\left(1 - \frac{L_H^l}{L_e\left(\frac{n_p A_e}{n_e A_p} - 1\right)}\right)}$$

Computations in panel b are based on the equation:

$$\frac{J_p}{J_p(0)} = f_p\left(1 + r + \frac{L_H^l}{L_e}\right) \Big/ \left(1 + f_p r + \frac{L_H^l}{L_e}\right)$$

$n_p A_e / n_e A_p = 4$, where A_e and A_p are the affinity of the redox and ATP hydrolysis reaction, respectively, and n_e and n_p are the H^+/e^- and H^+/ATP stoichiometry, respectively (cf. Ref. 8 for more details).

gives rise to a decrease in the rate of ATP synthesis. As a result, as can be seen in Fig. 2 (see the tangents to each curve at $f = 1$), at each value of r, the flux control of both pumps, but to a much larger extent that of the redox pumps, is increased with respect to the case with a negligible leak. A given concentration of redox inhibitor (and also, but to a lower extent, that of an ATP synthase inhibitor) gives rise to a larger inhibition of the rate of ATP synthesis. Moreover, the differences in the titration curves for different values of r are

reduced with respect to the case with a negligible leak.

The analysis of single inhibitor titrations performed above on the basis of a very simple linear chemiosmotic model is relevant for a correct use of these titrations as a means of discrimination between delocalized and localized models of free energy coupling. The finding of an inhibition of the rate of ATP synthesis proportional, for example, to the fraction of inhibited ATP synthases, i.e., of a flux-control coefficient equal to 1 for the ATP synthases, without the knowledge of the flux controls of the redox pumps and of the leak, does not allow to reach any conclusion about the localization or delocalization of the system. This result is perfectly compatible with a delocalized chemiosmotic system provided that the conductance of the ATP synthases is sufficiently low and/or the leak conductance is sufficiently high with respect to that of the redox pumps (i.e., r is sufficiently small and/or L^{l}_{H}/L_{e} is sufficiently high, cf. Figs. 1 and 2).

Fig. 2 shows that in the presence of a sufficiently high leak even the finding of an inhibition of the rate of ATP synthesis proportional to both the fraction of inhibited redox pumps and to that of inhibited ATP synthases, i.e., of a flux-control coefficient equal to 1 for both pumps, may be compatible with a delocalized chemiosmotic model, particularly when r is low, i.e., when the conductance of the ATP synthases is lower than that of the redox pumps. The finding which would be unequivocally in contrast with a delocalized model is the violation of the flux-control summation theorem (Eqn. 2), i.e., the finding that the sum of the flux control coefficients of the two pumps and of the leak is higher than 1 [18]. In the absence of a direct measure of the flux-control coefficient of the leak, which is difficult to perform (see, however, Ref. 16), the finding that the sum of the flux control coefficients of the two pumps is much higher than 1 is an unequivocal evidence against a delocalized model only if it can be shown that the leak conductance is negligible with respect to the conductances of the pumps.

In conclusion, if one wants to discriminate between delocalized and localized models of free-energy coupling on the basis of the results of single-inhibitor titrations in submitochondrial particles or chromatophores preparations it is necessary to measure: (1) the relative inhibition of the rate of ATP synthesis by both an inhibitor of the redox pumps and an inhibitor of the ATP synthases as a function of the fraction of inhibited enzymes, i.e., the flux-control coefficient of both pumps; and (2) either the flux-control coefficient of the leak or the value of the leak conductance of the coupling membrane with respect to the conductances of the pumps.

These conclusions have been reached assuming linear flow-force relationships for the three elements (redox pump, ATP synthase and leak) of the chemiosmotic protonic circuit. The relations between rate of electron transfer and $\Delta\tilde{\mu}_{H}$ and between rate of ATP synthesis and $\Delta\tilde{\mu}_{H}$ measured in many energy-transducing systems [22–27] and also those simulated on the basis of simple kinetic models of ion pumps [28–31] are approximately linear only in limited ranges of $\Delta\tilde{\mu}_{H}$.

However, the control theory analysis shows that the conclusions reached above assuming linear flow-force relations hold exactly also for the case with non linear flow-force relations (i.e., they are independent of the kinetics of the pumps), when the comparison is made on the relative inhibitions of the rate of ATP synthesis as induced by low concentrations of inhibitors of the redox and the ATP synthase pumps [18]. In fact the expressions for the flux-control coefficients of the pumps and of the leak as a function of what we have called their conductances in the chemiosmotic circuit, L_{e}, L_{p}, L^{l}_{H}, have been derived by Westerhoff and colleagues on the basis of two fundamental theorems of the control theory (the summation and the connectivity theorems, see Refs. 10, 12 and 18) which hold independently of the nature of the relations (whether linear or non-linear) between flows and $\Delta\tilde{\mu}_{H}$. Clearly, with non-linear relations the conductances, L's (or 'readinesses' with which the proton flows, J_{H}, change in response to a change in $\Delta\tilde{\mu}_{H}$), are not constant but are functions of $\Delta\tilde{\mu}_{H}$. As shown elsewhere [9], this dependence of the L coefficients on $\Delta\tilde{\mu}_{H}$, when the flow-force relations are non linear, has very important consequences for the correct interpretation of the results of double inhibitor titrations, since it can make compatible with a delocalized model results that with linear relations would be

unequivocally incompatible with it. In contrast, in the different experimental approach used in the present study, the conclusions reached above do not depend on the kinetics of the pumps (and are therefore model independent), provided that the comparison of the effect of the two inhibitors is done at the same value of $\Delta\tilde{\mu}_H$. Since the two inhibitors have opposite effects on $\Delta\tilde{\mu}_H$, if one wants to extend the conclusions reached on the basis of the linear model to the case with non linear relations, the comparison has to be done at the beginning of the titration curve, i.e., at very low concentrations of inhibitors.

Non-linear relations between flows and $\Delta\tilde{\mu}_H$ similar to those determined experimentally can be introduced in the chemiosmotic circuit by adopting for each pump the six-state kinetic model studied by Pietrobon and Caplan [31] with appropriate parameters in the simulations [9,32]. Fig. 3 shows titration curves obtained with this non linear kinetic model of chemiosmotic coupling (case C in Ref. 9) for different degrees and conditions of coupling.

The curves labelled 1 are obtained when, in the steady state without inhibitors (State 3), the leak conductance is negligible with respect to the conductances of the pumps at the same $\Delta\tilde{\mu}_H$, as is certainly the case in mitochondria. Mitochondria cannot be used as experimental system in the inhibitor titrations approach, since the interpretation of the results would be complicated by the presence of substrates translocators such as the ATP/ADP translocator, the dicarboxylic acid carrier etc. In previous studies, and also in the present work, submitochondrial particle (SMP) preparations have been used (or in photophosphorylating systems chromatophore preparations). However, as can be judged from the values of the respiratory control and P/O ratios of submitochondrial particles and chromatophores (see Table I), these preparations are loosely coupled with respect to mitochondria.

The curves labelled 2 in Fig. 3 are the titration curves obtained with the non-linear model by assuming that the loose coupling is due to an increased leak through the membrane, and selecting a value of leak conductance in the simulations which give rise to P/O and respiratory control ratios similar to those measured in our SMP preparation (values in parenthesis in Table I). With these coupling parameters a relative inhibition of the rate of ATP synthesis almost equal to the fraction of inhibited ATP synthases and even higher than the fraction of inhibited redox pumps is obtained. The sum of the flux-control coefficients of the two pumps is much higher than 1 (a result that intuitively seems in contrast with a delocalized model), since there is a high negative flux-control of the leak. In this particular case a non-ohmic leak ($J_{H,l} = L_H^{nl} \sinh \Delta\tilde{\mu}_H$) has been assumed, but essentially the same results can be obtained with an appropriate ohmic leak.

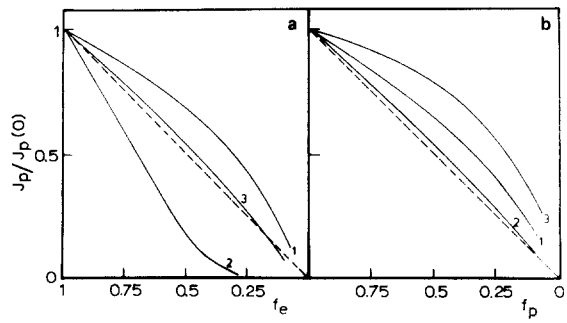

Fig. 3. Simulations of the relative rate of ATP synthesis, $J_p/J_p(0)$, as a function of the inhibition factor of the redox pumps, f_e (a) and of the inhibition of the ATPase pumps, f_p (b). The simulations are obtained with the nonlinear kinetic model of chemiosmotic coupling constituted by a redox proton pump model and an ATPase pump model as those described by Pietrobon and Caplan [31]. The third element of the model is a leak, which is assumed either ohmic with the conductance L_H^l or non-ohmic (in the latter case $J_{H'l} = L_H^{nl} \sinh \Delta\tilde{\mu}_H$). The kinetic parameters for the proton pump models are as in case C of Pietrobon and Caplan [9]. The affinities of the redox and ATP hydrolysis reactions are kept constant at 19.66 kcal·mol^{-1} and 25 kJ·mol^{-1}, respectively. ΔpH is kept constant at zero and therefore $\Delta\tilde{\mu}_H \equiv \Delta\psi$. Curves 1: $L_H^l = 0.5$ mol·kcal^{-1}·s^{-1}; the rate of ATP synthesis and the P/O ratio in the absence of inhibitors ($f_e = f_p = 1$) are $J_p(0) = 4.20$ s^{-1}; $(P/O)_0 = 2.29$; $(RCR)_0 = 22$. Essentially the same titration curves and values of $J_p(0)$, $(P/O)_0$ are obtained in the presence of a non-linear leak with $L_H^{nl} = 0.06$. By introducing in the proton-pump models the small degree of intrinsic uncoupling which allows to simulate the behaviour of mitochondria in static head [33–35] the titration curves 1 remain essentially unchanged, but $J_p(0) = 4.0$ s^{-1}; $(P/O)_0 = 1.77$; $(RCR)_0 = 7.07$. Curves 2: $L_H^{nl} = 1.4$; $J_p(0) = 1.21$ s^{-1}; $(P/O)_0 = 0.27$; $(RCR)_0 = 1.45$. Curves 3: fraction of uncoupled ATPases, $f_a^{unc} = 0.5$; $L_H^l = 0.5$ mol·kcal^{-1}·s^{-1}; $J_p(0) = 1.0$ s^{-1}; $(P/O)_0 = 0.28$. The values of the 'coupling parameters' (L_H^{nl} and f_a^{unc}) for the different mechanism of uncoupling were selected so as to give computed values of the P/O and RCR in the absence of inhibitors close to the experimental values: $(P/O)_0 = 0.3$, $(RCR)_0 = 1.5$.

It is possible that most of the leak is through the ATP synthases rather than through the membrane as a consequence of either loss of F_1 or intrinsic uncoupling (slip) of the pumps [27,33 and 34]. The curves labelled 3 in Fig. 3 are the titration curves obtained, assuming that the loose coupling of the SMP preparation is due to a fraction of completely uncoupled ATP synthases *.

Because of the low affinity of the ATP hydrolysis reaction, these uncoupled ATP synthases essentially translocate protons without coupled ATP synthesis, thus acting as a highly non-ohmic leak [31]. However, in contrast with a leak through the membrane this type of uncoupling gives rise to a lower relative inhibition of the rate of ATP synthesis by a given concentration of inhibitor with respect to the coupled case and decreases instead of increasing the flux control of the ATP synthases. The reason is that in this case the leak is intrinsic to the pump and thus any change in the concentration of the pumps brings about also a change in the amount of leak. Moreover, in the model the effect of decreasing the dissipative proton flow through the pumps prevails over that of decreasing the number of active ATP synthetases. This result holds if the inhibitor does not discriminate between the two fractions, i.e., it binds with the same affinity to both coupled and uncoupled pumps. Fig. 3 shows that, as in the case of a leak through the membrane, a fraction of uncoupled ATPases increases the flux control of the redox pumps and gives rise to a higher relative inhibition of the rate of ATP synthesis by a given concentration of redox inhibitor. However, in this case the sum of the flux-control coefficients of the two pumps does not significantly change.

Essentially the same results are obtained if it is assumed that the loose coupling is due to an increased intrinsic uncoupling of the entire population of ATP synthases. In this case, however, for the same uncoupling as measured from the values of the P/O and respiratory control ratios, the changes in the flux-control coefficients of the two pumps (their sum remaining still essentially the same) are smaller than in the presence of a fraction of completely uncoupled ATP synthases (not shown).

If it is assumed that the loose coupling is due to an increased intrinsic uncoupling of the redox pumps, titration curves close to the curves labelled 1 (corresponding to the tightly coupled case) are obtained (not shown). At the high affinity of the redox reaction at which the simulations are performed an increase of the rate constants of the slip transition in the model increases the probability of the uncoupled cycle that transfer electrons without pumping protons (redox slip), while the probability of the other uncoupled cycle (the proton slip that translocate protons down the gradient without reversing the redox reaction) remains very small [31,35].

Another possible reason for the loose coupling of the SMP preparation may be the presence of a fraction of highly leaky (or broken) vesicles in which the value of $\Delta\tilde{\mu}_H$ is below the threshold necessary to make ATP and the respiratory chains transfer electrons at maximum rate [36]. In this case the titration curves are exactly the same as in tightly coupled vesicles (curves labelled 1). This result holds as long as the inhibitors do not discriminate between coupled and uncoupled vesicles, i.e., they bind with the same affinity to both populations of vesicles.

The analysis of the simulations in Fig. 3 shows that for a correct interpretation of the results of the single inhibitor titrations in SMP's it is essential to understand the reason for the loose coupling of this preparation (cf. Table I), since different mechanisms of uncoupling lead to different behaviour. The modelling study illustrated below suggests an experimental approach to achieve this essential information.

* The six-state kinetic model of proton pump with which the flow-force relationships of the non-linear model are simulated has in it a 'slip' transition (see Ref. 31) that allows uncoupled cycles to occur (e.g., in the case of the ATPsynthase, cycles that translocate protons without making ATP or, in the reverse reaction, cycles that hydrolyze ATP without proton pumping), thus introducing the possibility of intrinsic uncoupling (slip) of the pumps. A fraction of completely uncoupled pumps is simulated by assigning very high values to the rate constants of the slip transition in the model in a certain fraction of pumps and low values in the remaining fraction, so that the probability of coupled cycles in one fraction is practically zero and in the other fraction is practically one. An increased intrinsic uncoupling is simulated by increasing the rate constants of the slip transition in all the pumps.

Figs. 4 and 5 show the simulated behaviour of the P/O ratio upon inhibition of the redox and ATP synthase pumps and of the respiratory control ratio, RCR, upon inhibition of the redox pumps for the different possible mechanisms of uncoupling discussed above. In each case the simulations are performed using the non linear kinetic model of chemiosmotic coupling and parameters such that each type of uncoupling mechanism gives rise to P/O ratios and RCR's, in the absence of inhibitors, close to those measured in our SMP preparation. For comparison, the behaviour of the P/O ratio and the RCR in the presence of a negligible leak is also shown (curve labelled 1).

Figs. 4 and 5 show that the P/O ratio and the RCR vary in a very different manner upon inhibition depending on the nature of the uncoupling mechanism. If the loose coupling of the SMP preparation is exclusively due to an increased leak conductance through the membrane (as that which gives rise to the titration curves labelled 2 in Fig. 3) both the P/O ratio and the RCR decrease

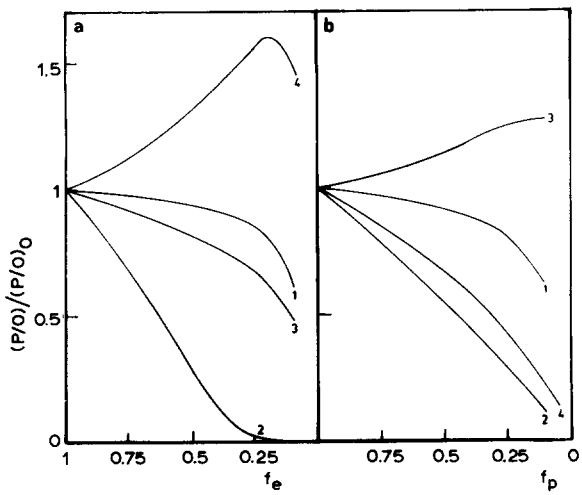

Fig. 4. Simulations of the relative P/O ratio, $(P/O)/(P/O)_0$, as a function of the inhibition factor of the redox pumps, f_e (a) and of the inhibition factor of the ATPase pumps, f_p (b). The simulations are obtained as in Fig. 3 with the same model and parameters. $P/O = 2J_p/J_e$, where J_p and J_e are the rate of ATP synthesis and of electron transfer, respectively, in the stationary state of phosphorylation at constant low affinity of the ATP hydrolysis reaction ($A_p = 6$ kcal·mol^{-1}). Curves 1, 2 and 3: 'coupling parameters' as in Fig. 3. Curves 4: fraction of uncoupled vesicles $f_{SMP}^{unc} = 0.7$; $L_H^1 = 0.5$ mol·kcal^{-1}·s^{-1}; $(P/O)_0 = 0.26$.

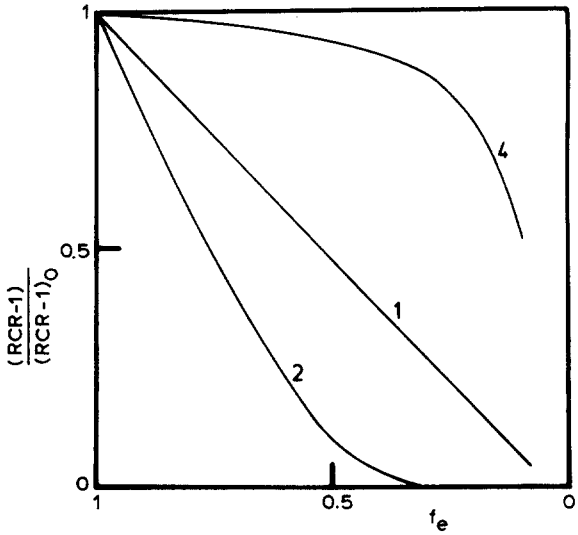

Fig. 5. Simulations of the relative normalized respiratory control ratio, $(RCR-1)/(RCR-1)_0$, as a function of the inhibition factor of the redox pumps, f_e. The simulations are obtained as in Fig. 3 with the same model and parameters. $RCR = J_e^{max}/J_e^{sh}$, where J_e^{max} is the maximal rate of electron transfer in the saturation region at low $\Delta\tilde{\mu}_H$ and J_e^{sh} is the rate of electron transfer in static head. Curves 1 and 2: 'coupling parameters' as in Fig. 3. Curve 4: fraction of uncoupled vesicles $f_{SMP}^{unc} = 0.7$; $L_H^1 = 0.5$ mol·kcal^{-1}·s^{-1}; $(RCR)_0 = 1.4$.

rapidly upon inhibition of the redox pumps, and the same happens for the P/O ratio upon inhibition of the ATP synthases (curves labelled 2 in Figs. 4 and 5).

On the other hand, if the increased leak is through a fraction of uncoupled (e.g., deprived of F_1) ATP synthases (as that which gives rise to the titration curves labelled 3 in Fig. 3) the P/O ratio remains relatively unchanged upon inhibition of the redox pumps and it increases upon inhibition of the ATP synthases (curves labelled 3 in Fig. 4). The small sensitivity of the P/O ratio to inhibition of the redox pumps is due to the fact that the dissipative proton flow through the ATP synthases is steeply dependent on $\Delta\tilde{\mu}_H$ and it rapidly decreases as inhibition proceeds as a consequence of the (even if small) decrease of $\Delta\tilde{\mu}_H$. The increase of the P/O ratio upon inhibition of the ATP synthases is due to the parallel inhibition of the leak together with the pumps, and to the fact that the effect of the decreased dissipative proton flow prevails over that of the decreased number of active ATP synthases. If the loose coupling is due

to an increased intrinsic uncoupling in the entire population of ATP synthases, the P/O ratio remains relatively insensitive to both inhibitors of the redox and ATP synthases pump (not shown), i.e., the behaviour is close to that shown in Fig. 4 for vesicles possessing a negligible leak (curves labelled 1).

The curves labelled 4 in Figs. 4 and 5 are obtained under the assumption that the loose coupling is due to inhomogeneity in the preparation, i.e., to the presence of a fraction of highly leaky (or broken) vesicles. A similar behaviour is obtained assuming that the loose coupling is due to intrinsic uncoupling of the redox pumps (not shown). In these cases the P/O ratio increases (at least in a certain range) upon inhibition of the redox pumps and decreases upon inhibition of the ATP synthases. The RCR remains relatively insensitive to inhibition of the redox pumps. The increase of the P/O ratio in a certain range of f_e is due to the fact that, in this range, the decrease in the rate of electron transfer is mainly due to inhibition of the uncoupled electron flow, which is more sensitive to a given concentration of inhibitor than the coupled one. This explains also the relative insensitivity of the RCR upon inhibition. On the other hand, when the ATP synthases are inhibited the decrease of the P/O ratio with f_p is due to the fact that the uncoupled electron flow remains unchanged.

The analysis of the simulations in Figs. 4 and 5 shows that an experimental determination of the P/O ratio and the RCR as a function of the fraction of inhibited pumps provides informations on the mechanism of uncoupling of the SMP preparations. These informations are essential for the correct interpretation of the results of single inhibitor titrations and more in general of experiments aiming at clarifying the energy coupling mechanism in SMP's.

Results

The ATPase and the redox proton pumps of SMP's have been titrated with inhibitors under two sets of experimental conditions. In one type of experiment (expt. a) the rate of ATP-driven NAD reduction by the first site of the respiratory chain was measured at increasing concentrations of DCCD (an inhibitor of the primary ATPase pumps) and of rotenone (an inhibitor of the secondary redox pumps). In the other type of experiment (expt. b) the rate of ATP synthesis was measured at increasing concentrations of antimycin (an inhibitor of the primary redox pumps) and of DCCD (which in this case inhibits the secondary ATP synthetases pumps).

For a correct interpretation of the results of these inhibitor titrations, utilizing the analysis described in the previous section, the fraction, f, of active (non-inhibited) pumps at each concentration of inhibitor must be known. The active fraction, f, can be obtained by measuring the relative inhibition of the spontaneous reaction catalyzed by each pump under uncoupled conditions (excess FCCP present). The maximal, $\Delta\tilde{\mu}_H$-uncontrolled rate of the spontaneous reaction measured at each concentration of inhibitor relative to that in the absence of inhibitor has been taken as equal to the fraction, f, of active pumps. If the binding of the inhibitor is not $\Delta\tilde{\mu}_H$ dependent and, when a secondary pump is titrated, if the binding of the inhibitor does not depend on the direction (whether uphill or downhill) in which the pump works, the fraction of active pumps at each concentration of inhibitor in State 3 (stationary state of phosphorylation in expt. b and of ATP-driven NAD reduction in expt. a) can be considered to be equal to the active fraction, f, measured in uncoupled conditions.

The inset in Fig. 6 shows the relative rate of uncoupled ATP hydrolysis, J_{ATP}, as a function of the concentration of DCCD. As expected for an irreversible inhibitor, in the absence of inactive binding sites [18], there is a range (in which the dissociation constant, K_D, is much lower than the total amount of free enzyme) where the relative inhibition of the uncoupled rate is proportional to the concentration of inhibitor. The relative rate of uncoupled ATP hydrolysis is used to calculate the fraction, f_p, of active ATPases at each concentration of DCCD. A control experiment has been performed to test the possibility that the natural inhibitor protein whose binding is $\Delta\tilde{\mu}_H$ dependent might interfere with the binding of DCCD and cause the fraction of active ATP synthetases at each concentration of DCCD to be different in State 3 with respect to that in the uncoupled state.

The rate of uncoupled ATP hydrolysis as a function of DCCD has been measured in a preparation of SMP's devoided of the natural inhibitor protein with the method suggested by Penefsky [37]. While the initial rate of ATP hydrolysis is about 7-times higher in the preparation without inhibitor protein the relative inhibition at each concentration of DCCD is the same for the two preparations (not shown).

Fig. 6 shows that the rate of ATP-driven NAD reduction titrated with DCCD is proportional to the fraction, f_p, of active ATPases. Therefore, the flux-control coefficient of the ATPases (the primary proton pumps) on the rate of ATP-driven NAD reduction is equal to 1.

The inset in Fig. 7 shows the uncoupled rate of NADH oxidation as a function of the concentration of rotenone, an inhibitor of the NADH-ubiquinone oxidoreductase complex. The relative rate of uncoupled electron transfer has been used to calculate the fraction of active redox pumps, f_e, at each concentration of rotenone. Fig. 7 shows that the rate of ATP-driven NAD reduction titrated with rotenone is proportional to the fraction, f_e, of active redox pumps. Therefore, also the flux-control coefficient of the redox pumps (the secondary proton pumps) on the rate of ATP-driven NAD reduction is equal to 1.

The inset in Fig. 8 shows the uncoupled rate of succinate oxidation as a function of the concentration of antimycin, an inhibitor of the bc_1 complex. The sigmoidicity of the titration curve or, in other words, the non-proportionality between the rate of uncoupled electron transfer and the concentration of an irreversible inhibitor such as antimycin, is due to the very low flux control coefficient of the bc_1 complex in the chain of enzymes which transfer electrons from succinate to oxygen. In fact it has been shown that, in these conditions, most of the control on the electron flow resides on the electron donor side of the Q pool [14]. Therefore, in this case, the relative rate of uncoupled electron transfer at each concentration of inhibitor is not equal to the fraction f_e of active redox pumps. If it could be shown that the distribution of flux control among the electron-transfer enzymes is not $\Delta\tilde{\mu}_H$, dependent one could still define the relative rate of uncoupled electron transfer from succinate to oxygen as an 'effective f_e'. The finding that the rate of ATP synthesis is proportional to this 'effective f_e' would still indicate that the flux control coefficient of the redox pumps on the rate of ATP synthesis is equal to 1. However, in the literature there are indications that the control

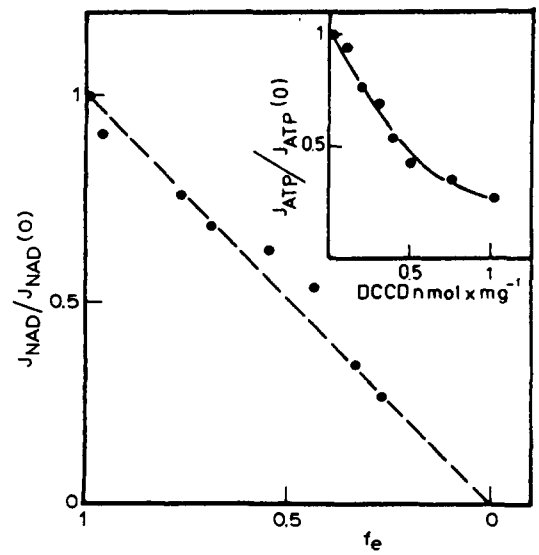

Fig. 6. Relative rate of ATP-driven NAD reduction, $J_{NAD}/J_{NAD}(0)$, as a function of the fraction of active ATPase pumps, f_p, in a titration with DCCD. Inset: Relative rate of uncoupled ATP hydrolysis, $J_{ATP}/J_{ATP}(0)$, as a function of the DCCD concentration. Submitochondrial particles were incubated in their suspension medium at the concentration of 25 mg·ml^{-1} with variable amounts of DCCD for 40 min at room temperature. The DCCD-treated submitochondrial particles were then incubated (0.125 mg·ml^{-1}) in a medium containing 0.13 M sucrose, 20 mM Tris/Mops, 5 mM $(NH_4)_2HPO_4$, 0.5 mM EGTA, 5 mM magnesium acetate, 20 mM succinate/Tris, 10 mM phosphocreatine, non-limiting amount of creatinekinase, 2 mM ATP, 300 ng·mg^{-1} antimycin. After 8 min of incubation, 0.5 mM NAD was added and the rate of NAD reduction was monitored spectrophotometrically. The rate of NAD reduction in the absence of DCCD, $J_{NAD}(0)$, was 75 nmol·min^{-1}·mg^{-1}. The fraction of non-inhibited ATPase pumps (f_p) was calculated from the relative inhibition by DCCD of the uncoupled rate of ATP hydrolysis (inset) as assayed in parallel samples. The DCCD-treated submitochondrial particles were incubated for 8 min in a medium containing 0.13 M sucrose, 20 mM Tris/Mops, 5 mM phosphate/Tris, 0.5 mM EGTA, 5 mM magnesium Acetate, 0.2 mM NADH, 1 mM phospho*enol*pyruvate, 0.1 mM Ap_5A, non-limiting amount of pyruvate kinase/lactate dehydrogenase, 4 μM rotenone, 300 ng per mg antimycin, 1.5 μmol per mg FCCP. The ATP hydrolysis was started by the addition of 2 mM ATP. The rate of uncoupled ATP hydrolysis in the absence of DCCD, $J_{ATP}(0)$, was 341 nmol·min^{-1}·mg^{-1}.

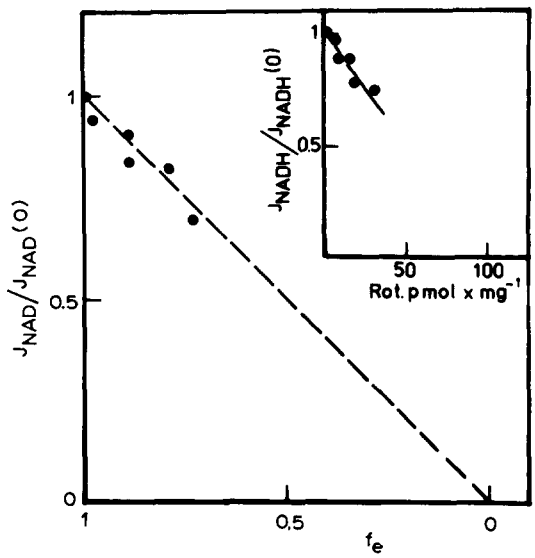

Fig. 7. Relative rate of ATP-driven NAD reduction, $J_{NAD}/J_{NAD}(0)$, as a function of the fraction of active redox pumps in a titration with rotenone. Inset: relative rate of uncoupled NADH oxidation, $J_{NADH}/J_{NADH}(0)$, as a function of the rotenone (Rot.) concentration. Submitochondrial particles were incubated (0.125 mg·ml^{-1}) for 8 min in the reaction medium as in Fig. 6 in the presence of variable concentrations of rotenone. The NAD reduction was started by the addition of 0.5 mM NAD and monitored spectrophotometrically. The rate of NAD reduction in the absence of rotenone, $J_{NAD}(0)$ was 75 nmol·min^{-1}·mg^{-1}. The fraction of non-inhibited redox pumps (f_e) was calculated from the relative inhibition of the rate of uncoupled NADH oxidation (inset) as assayed in parallel samples at the same protein and rotenone concentrations and in the same medium except for the absence of succinate and antimycin and the presence of 5 μg·mg^{-1} oligomycin and 1.5 μg·mg^{-1} FCCP. After 8 min of incubation NADH oxidation was started by the addition of 0.3 mM NADH. The maximal rate of NADH oxidation in the absence of rotenone, $J_{NADH}(0)$, was 190 nmol·min^{-1}·mg^{-1}.

of the bc_1 complex on the rate of electron transfer is indeed $\Delta\tilde{\mu}_H$ dependent * [38]. This eliminates the potential utility of defining an 'effective f_e', thus making it impossible to draw any clearcut conclusion from an experiment in which the succinate-driven rate of ATP synthesis is inhibited with antimycin.

Fig. 8 shows the uncoupled rate of durohydroquinone oxidation as a function of antimycin. With this substrate the bc_1 complex gains complete control on the rate of electron transfer [14]. The relative inhibition of the uncoupled rate is proportional to the concentration of inhibitor and can be used to calculate the fraction of active redox pumps at each concentration of antimycin. The titrations of the rate of ATP synthesis with antimycin and DCCD using durohydroquinone as electron donor are shown in Fig. 9. Fig. 9 shows that the durohydroquinone-driven rate of ATP synthesis is proportional to both the fraction of active redox and ATP synthetase pumps. The flux

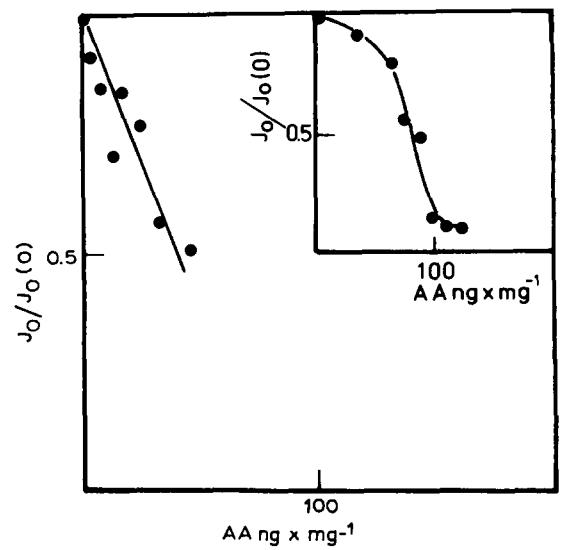

Fig. 8. Titration with antimycin of the uncoupled respiration with durohydroquinone as electron donor. Inset: titration with antimycin of the uncoupled respiration with succinate as electron donor. Submitochondrial particles (0.3 mg·ml^{-1}) were incubated in the presence of variable amounts of antimycin in a medium containing: 0.13 M sucrose, 20 mM Tris/Mops, 5 mM magnesium Acetate, 5 mM $(NH_4)_2HPO_4$, 5 mM phosphate/Tris, 5 μg per mg oligomycin, 0.5 mM EGTA, 4 μM rotenone and the respiratory substrate (either durohydroquinone or succinate). The durohydroquinone was generated by reduction of duroquinone (50 μM) with NADH (2 mM) and non limiting amount of diaphorase. After 8 min FCCP (1.5 μmol·mg^{-1}) was added and the respiratory rate measured. The maximal rate of respiration in the absence of inhibitor, $J_O(0)$, was 101 ngatom·min^{-1}·mg^{-1} with durohydroquinone (50 μM) and 354 ngatom·min^{-1}·mg^{-1} with succinate (20 mM) as electron donor. AA, antimycin A.

* As pointed out by Stoner [38], the fact that the titration curve with antimycin of the mitochondrial respiration in static head has a lower sigmoidicity with respect to that in the uncoupled state, and the consequent increase of the respiratory control ratio in a certain range [33] may well be expression of an increase of the flux control coefficient of the bc_1 complex as $\Delta\tilde{\mu}_H$ increases.

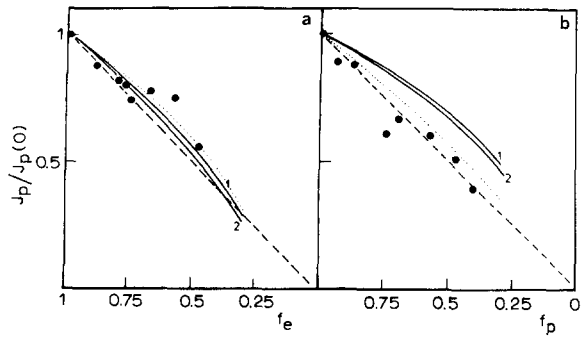

Fig. 9. Relative rate of ATP synthesis, $J_p/J_p(0)$, as a function of (a) the fraction of active redox pumps, f_e, in a titration with antimycin and (b) the fraction of active ATPase pumps, f_p, in a titration with DCCD. Medium as in Fig. 8 (with durohydroquinone as electron donor), except for the absence of oligomycin and the presence of 0.1 mM Ap_5A, 10 mM glucose and non-limiting amount of hexokinase. After 8 min in the presence of variable concentrations of either antimycin or DCCD, the ATP synthesis was started by the addition of 0.5 mM ADP. The rate of ATP synthesis in the absence of inhibitors, $J_p(0)$, was 62 nmol·min^{-1}·mg^{-1}. The fraction of non-inhibited redox pumps, f_e was calculated from the relative inhibition of the rate of uncoupled respiration measured in the presence of 1.5 μmol·mg^{-1} FCCP; the fraction of non-inhibited ATPase pumps, f_p, was calculated from the relative inhibition of the uncoupled rate of ATP hydrolysis as described in Fig. 7. The curves are simulations obtained with the same model and kinetic parameters as those in Fig. 3, but with a lower constant value (18.1 kcal·mol^{-1}) of the affinity of the redox reaction. The 'coupling parameters' selected in each case are such to give computed values of the P/O and RCR in the absence of inhibitors close to the experimental values measured with 50 μM durohydroquinone as electron donor: $(P/O)_0 = 0.6$, $(RCR)_0 = 1.5$. (———1) fraction of uncoupled vesicles $f_{SMP}^{unc} = 0.55$, $L_H^l = 0.5$ mol·kcal^{-1}·s^{-1}, $(P/O)_0 = 0.60$, $(RCR)_0 = 1.39$; (———2) $f_{SMP}^{unc} = 0.5$, $L_H^{nl} = 0.12$, $(P/O)_0 = 0.61$, $(RCR)_0 = 1.36$; (·····) $f_{SMP}^{unc} = 0.13$, $L_H^{nl} = 0.12$, $(P/O)_0 = 0.60$, $(RCR)_0 = 1.86$, number of ATPase pumps lowered by a factor 0.25. In all the simulations in the proton pump models there was the small intrinsic uncoupling equal to that used to simulate the static head behaviours of mitochondria [35].

control coefficient on the rate of ATP synthesis is then equal to 1 for both redox and ATP synthetase pumps. The same proportional inhibition pattern shown in Figs. 6, 7 and 9 has been observed in six experiments performed in different SMP preparations. We can then confidently conclude that our results show a flux control coefficient equal to 1 for both primary and secondary pumps of oxidative phosphorylation.

We have pointed out in the previous section that this type of result (i.e., that the sum of the flux control coefficients of the two primary and secondary proton pumps is much higher than 1) can be considered an unequivocal evidence against a delocalized model of energy coupling if it can be shown that the membrane proton conductance is negligible with respect to the conductances of the pumps. In mitochondria this is certainly true, as one can immediately ascertain from a comparison between the measured membrane conductance and the flow-force relationships [26,39]. However, submitochondrial particles are loosely coupled preparations with respect to mitochondria, as shown by the low P/O ratios and RCR's in Table I. It is then essential to establish if an increased membrane proton conductance is responsible for the low P/O ratio and RCR of submitochondrial particles.

We have attempted to measure directly the dissipative proton efflux upon inhibition with antimycin of SMP's incubated in the presence of K^+ (30 mM) and valinomycin. The measured proton efflux (60 nmol·min^{-1}·mg^{-1}) is more than one order of magnitude lower than the rate of respiration in static head multiplied by the H^+/O stoichiometry (1560 nmol·min^{-1}·mg^{-1} with a stoichiometry $H^+/O = 6$) at similar $\Delta\tilde{\mu}_H$. The experiment suggests that the high rate of respiration in static head can be accounted for only to a very limited extent by the dissipative proton efflux. A low value of dissipative proton flux as measured by ion fluxes after proton pump inhibition can be due to artifactual underestimations. We have therefore searched for independent approaches to establish the role of the leaks in causing apparent loose coupling of SMP's.

We have shown in the previous section that the behaviour of the P/O ratio and the RCR upon inhibition of the proton pumps can be very informative about the nature of the uncoupling mechanism. Fig. 10 shows the P/O ratio, i.e., the ratio between the rate of ATP synthesis and the rate of respiration (with durohydroquinone as electron donor), as a function of the fraction of active redox pumps, f_e, and ATP synthetases, f_p, in titrations with antimycin and DCCD, respectively. In the titration with antimycin the P/O ratio remains approximately constant or even slightly increases up to a 50% inhibition of the redox pumps. In contrast, in the titration with DCCD

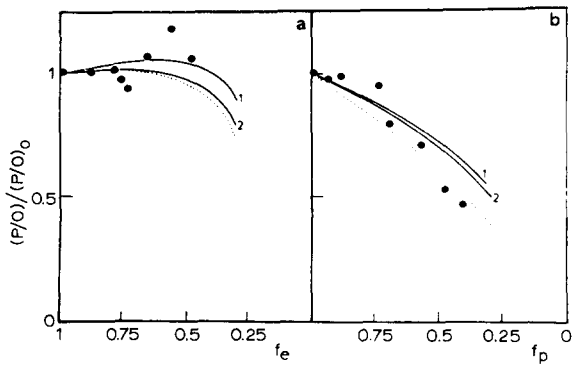

Fig. 10. Relative P/O ratio, $(P/O)/(P/O)_0$, as a function of (a) the fraction of active redox pumps, f_e, in a titration with antimycin and (b) the fraction of active ATPase pumps, f_p, in a titration with DCCD. The P/O ratio is calculated as the ratio between the rate of durohydroquinone-driven ATP synthesis and the State 3 respiration rate measured in parallel samples under the same conditions as described in Fig. 9. The P/O ratio, $(P/O)_0$, in the absence of inhibitor was 0.6. The curves are simulations obtained with the same model and kinetic parameters as those in Fig. 3, but with lower constant value (18.1 kcal·mol^{-1}) of the affinity of the redox reaction. The 'coupling parameters' of the simulations are as in Fig. 9.

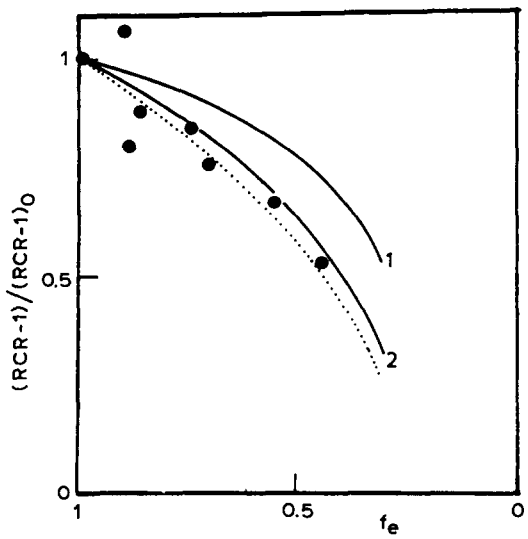

Fig. 11. Relative normalized respiratory control ratio, $(RCR-1)/(RCR-1))_0$, as a function of the fraction of active redox pumps, f_e, in a titration with antimycin. Medium and conditions as in Fig. 8 with durohydroquinone as electron donor. RCR is calculated as the ratio between the respiratory rate in the presence of excess FCCP, J_o^{unc}, and the respiratory rate measured before the addition of FCCP, J_o^{sh}. The respiratory control ratio without inhibitor, $(RCR)_0$, was 1.5. The curves are simulations obtained as in Fig. 10.

the P/O ratio decreases almost proportionally to the fraction of inhibited ATP synthetases. Fig. 11 shows the respiratory control ratio, RCR, as a function of the fraction of active redox pumps in the titration with antimycin. The RCR decreases almost proportionally to the fraction of inhibited redox pumps.

A very interesting observation, which also gives information on the mechanism of uncoupling of SMP's, is reported in Table II: the P/O ratio measured with a low (limiting) concentration of durohydroquinone as electron donor (supporting a maximal rate of respiration, in the presence of excess FCCP, 3.5-times lower than that obtained with excess succinate) is always higher (about two times) than the P/O ratio measured with excess succinate as electron donor. The RCR measured with the two substrates is similar.

Discussion

We have found that in submitochondrial particles the relative inhibition of the rate of ATP synthesis and also of ATP-driven NAD reduction is equal to both the fraction of inhibited redox pumps (in titrations with either antimycin or rotenone; Figs. 9a and 7) and the fraction of inhibited ATPases (in titrations with DCCD; Fig. 9b and 6). This means that both the primary and the secondary proton pumps of oxidative phosphorylation have a flux-control coefficient on the rate of the endergonic reactions equal to one, i.e., they are both 'completely rate limiting'.

The observation previously reported by the group of Slater [40] that the relative inhibition of the NADH-driven ATP synthesis in SMP's is proportional to both the relative inhibition of the uncoupled ATP hydrolysis and the State 3 NADH oxidation leads to the same conclusion. In fact in SMP's the relative inhibition by rotenone of the state 3 NADH oxidation is equal to the relative inhibition of the uncoupled NADH oxidation and can then be considered equal to the fraction of inhibited redox pumps. An inhibition of the rate of ATP synthesis proportional to both the fractions of inhibited redox and ATPase pumps has been previously reported also in chromatophores (Ref. 41, cf., however, Ref. 42).

The analysis presented in the Theory sections

shows that the finding of a flux control coefficient equal to 1 for both the redox and the ATPase pumps is incompatible with the delocalized chemiosmotic model of free-energy coupling if the proton conductance of the coupling membrane is sufficiently low with respect to the conductances of the proton pumps. In order to reach a clearcut conclusion about the mechanism of energy coupling on the basis of the single inhibitor titrations it has been essential to ascertain the origin of the well-known loose coupling of the submitochondrial particles preparations, which have very low RCR and P/O ratios and very high rates of respiration in static head with respect to mitochondria (see Table I).

Three independent types of observation show that the loose coupling of our preparation is not mainly due to an increased membrane proton conductance: (i) the high rate of respiration in static head is accounted for only to a minimal extent by the dissipative proton efflux measured upon inhibition with antimycin; (ii) the P/O ratio remains constant or even increases slightly up to a 50% inhibition of the redox pumps; if the loose coupling is due to a high leak the P/O ratio would rapidly decrease as shown by simulation 2 in Fig. 4a; (iii) the P/O ratio measured with a limiting quantity of durohydroquinone as electron donor is higher than the P/O ratio measured with excess succinate as electron donor. The respiratory control ratio is similar in the two cases. If the loose coupling were due to a high membrane conductance, both the P/O ratio and the RCR would be higher with excess substrate (high affinity) than with a limiting substrate concentration (low affinity). This is shown by the computed values of RCR and P/O in the third and fourth row of Table II).

These independent observations lead to the conclusion that the loose coupling of our preparation is not due to high leak, and raise two questions: (1) what is the origin of the uncoupling of SMP's with respect to mitochondria? and (2) how does the uncoupling affect the single-inhibitor titrations? We have answered these questions also with the help of a comparison between experimental observations and simulations. These have been carried out by using the non-linear kinetic coupling of chemiosmotic model of Pietrobon and Caplan [9].

TABLE I

RESPIRATORY CONTROL RATIOS AND P/O RATIOS REPORTED IN THE LITERATURE FOR RAT-LIVER MITOCHONDRIA, BOVINE HEART SUBMITOCHONDRIAL PARTICLES (SMP's) AND *RHODOPSEUDOMONAS SPHAEROIDES* CHROMATOPHORES

In the cases of mitochondria and SMP's lower and upper values reported in the literature are shown. The values in parentheses are those measured in our SMP preparations. The values for chromatophores are taken from Clark et al. [27] and Myatt and Jackson [43].

	$\frac{J_e^{ADP}}{J_e^{sh}}$	P/O	$\frac{J_e^{unc}}{J_e^{sh}}$
Mitochondria	5–6	1.4–1.8	7 –10
SMP's	1–1.2 (1.1)	0.3–1.2 (0.3)	1.3– 2 (1.5)
Chromatophores	1.2	0.77	6

TABLE II

EXPERIMENTAL AND COMPUTED VALUES OF P/O RATIOS AND RCR AT LOW AND HIGH AFFINITY OF THE REDOX REACTION

With 50 μM durohydroquinone the maximal rate of respiration (in the presence of excess FCCP) is 3.5-times lower than the maximal rate of respiration in the presence of excess (20 mM) succinate. The values in the last four rows are computed with parameters in the kinetic model of chemiosmotic coupling as in Fig. 3 and with the affinity of the redox reaction kept constant at 18.1 and 19.66 kcal·mol^{-1} for the low-affinity and high-affinity cases, respectively. The computed maximal rate of electron transfer when the affinity is kept constant at 18.1 kcal·mol^{-1} is 3.5-times lower than the maximal rate of electron transfer with the affinity kept constant at 19.66 kcal·mol^{-1}. For the different mechanisms of uncoupling the parameters (indicated in the first column) were selected so as to give computed values of P/O ratio and RCR at low affinity close to the experimental values measured with 50 μM durohydroquinone as substrate.

	P/O		RCR	
	low affinity	high affinity	low affinity	high affinity
50 μM durohydroquinone	0.6		1.5	
20 mM succinate		0.3–0.4		1.5
$L_H^{nl} = 0.39$	0.60	1.00	1.28	3.05
$L_H^{l} = 2.25$	0.61	1.13	1.35	3.64
$f_{SMP}^{unc} = 0.55$	0.60	0.40	1.39	1.63
$f_{SMP}^{unc} = 0.5$; $L_H^{nl} = 0.12$	0.61	0.44	1.36	1.69

The modeling approach has indicated the possibility of achieving information about the origin of the loose coupling phenomenon in the SMP preparation from the analysis of the experimental pattern of the P/O ratio and the RCR upon redox and ATPase inhibition, and has then led us to perform the experiments shown in Figs. 10 and 11. In order to relate the P/O and the RCR to the fraction of active pumps, f_e, we have used durohydroquinone as electron donor, which allows to derive f_e from the relative rate of uncoupled electron transfer. The use of a low concentration of durohydroquinone (giving a maximal rate of respiration 3.5-times lower than the maximal rate achieved with excess succinate) has proved to be useful in many respects. We have observed that the P/O ratio in the presence of low concentrations of durohydroquinone is higher than the P/O in the presence of excess succinate (while the RCR is similar with the two substrates). Such an interesting observation adds to the experimental patterns of the P/O ratios as a function of f_e and f_p and of the RCR as a function of f_e as a fourth independent result which has to be accounted for by any proposed mechanism for the SMP loose coupling.

Table II shows that among the mechanisms analyzed only the inhomogeneity, i.e., the presence of a subpopulation of highly leaky, non-phosphorylating vesicles together with a fraction of well-coupled vesicles, gives rise in the chemiosmotic model to a P/O ratio which is lower at high affinity than at low affinity of the redox reaction and to similar RCR's at the two affinities. In fact, with an inhomogeneous SMP preparation, the measured P/O ratio, i.e., the ratio between the rates of ATP synthesis and of respiration (J_p/J_o), is low because J_o is given by the sum of the State 3 respiratory rate of the coupled vesicles and the (maximal) respiratory rate of the uncoupled vesicles. When the concentration of the electron donor and therefore the affinity of the redox reaction is lowered (so as, for example, to give as in the simulations and in the experiments a maximal rate of respiration 3.5 times lower) the contribution of the leaky vesicles to the measured respiratory rate diminishes, while the ratio J_p/J_o provided by the coupled vesicles remains unchanged due to the smallness of the leak. As a consequence, the measured P/O ratio is higher at low than at high affinity. In contrast, an homogeneous increase of the leak in the entire population of vesicles results in a depression of the P/O ratio which is the more marked the lower is the conductance of the redox proton pumps with respect to the leak conductance, i.e., the lower is the affinity.

The assumption that the loose coupling of the SMP's is mainly due to the presence of a subpopulation of highly leaky vesicles is also the only one which allows to simulate closely the measured constancy of the P/O ratio upon redox inhibition and the decrease of the P/O ratio upon inhibition of the ATP synthases. We have already pointed out that the constancy of the P/O ratio upon inhibition of the redox pumps (Fig. 4a) argues against an uncoupling due to a high leak through the membrane. On the other hand the decrease of the P/O ratio upon inhibition of the ATP synthases (Fig. 4b) argues against an uncoupling mainly due to a high leak through the ATPases devoid of F_1. The simulation labelled 3 in Fig. 4b shows that, if the inhibitor inhibits with the same efficiency the coupled and uncoupled ATPases (and therefore the leak), in the presence of a fraction of uncoupled ATPases the P/O ratio tends to increase upon inhibition. Also, the fact that the high rate of respiration in static head of the SMP's is only 5–10% inhibited by an excess of either DCCD or oligomycin suggests that the leak through F_0 devoid of F_1 does not significantly contribute to the loose coupling of the SMP preparation.

The curves labelled 1 in Figs. 10 and 11 are the simulations obtained at low affinity under the assumption that the low P/O ratio and RCR are due to the presence of a subpopulation of highly leaky vesicles (where the rate of ATP synthesis is zero and the rate of electron transfer is maximal). It is seen that, at low affinity, the P/O ratio increases only slightly (Fig. 10a, curve 1), at variance from the simulation at high affinity where the P/O ratio increases markedly upon inhibition of the redox pumps (curve 4 in Fig. 4a). The reason for the different behaviour at the two affinities of the redox reaction becomes clear after noting that the rate of electron transfer of the coupled vesicles in the stationary state of phosphorylation (State 3) is close to the maximal

rate at low affinity, and well below at high affinity. Therefore at low affinity the rates of electron transfer of the coupled and uncoupled vesicles are almost equally sensitive to a given concentration of inhibitor, while at high affinity the rate of electron transfer of the uncoupled vesicles is more sensitive to the inhibitor than the rate of the coupled vesicles.

Fig. 10 shows that the assumption of the loose coupling of the SMP preparation being mainly due to the presence of a subpopulation of highly leaky vesicles leads to a good correspondence between simulated (curve 1) and experimental behaviour of the P/O ratio upon redox or ATPase inhibition. By slightly increasing the leak in the population of coupled vesicles also the behaviour of the RCR upon redox inhibition can be closely simulated (curves labelled 2 in Figs. 10 and 11).

We conclude that the presence of a subpopulation of leaky non phosphorylating vesicles is the main cause of the low P/O ratio and RCR in the SMP preparation *. The titration curves in Fig. 9 then reflect the behaviour of the population of coupled phosphorylating vesicles. The question now becomes: is the leak in this population sufficiently high to account for a flux control coefficient equal to one for both pumps without the need of rejecting the delocalized mechanism of energy coupling?

The measured constancy of the P/O ratio upon redox inhibition in the presence of a limiting quantity of redox substrate argues in favor of a small leak conductance in the population of coupled phosphorylating vesicles. In fact, the presence of a subpopulation of highly leaky vesicles is not expected to give rise to an increase of the P/O ratio upon redox inhibition in the experiments with low concentration of durohydroquinone. At these concentrations the State-3 respiration rate is close to the maximal rate (as judged from control experiments performed with mitochondria). The P/O ratio is then expected to decrease upon redox inhibition if a significant leak is present in the coupled vesicles. Moreover, note that in the presence of a limiting quantity of substrate, which, by definition increases the flux control of the redox pumps and decreases that of the ATPases, it is very unlikely that even a high leak can give rise to the observed unitary flux control coefficients for both primary and secondary pumps. The simulations in Fig. 2 (as well as those performed with the nonlinear model) show that only when the conductance of the ATP synthases is much lower than that of the redox pumps (i.e., only when, with a negligible leak, most of the control of the rate of ATP synthesis is at level of the ATP synthases, which is certainly not the case in our experiments) a high leak can lead to a flux control coefficient equal to 1 for both pumps.

These conclusions are fully supported by the comparison between simulations and experiments. Only the assumption that the population of phosphorylating vesicles has a low proton conductance allows to simulate the constancy of the P/O ratio upon inhibition with antimycin (Fig. 10) and a P/O value lower with excess succinate than with limiting durohydroquinone (Table II). The conductance value which leads to the best fit between simulations and experiments is only 2.4-times higher than that which has been used to simulate the static head behaviour of mitochondria [35]. Not surprisingly, with this conductance, the simulated titration curves (curve 2) do not fit the data in Figure 9. It is important to note that this

* There is actually an additional possible explanation for the loose coupling not yet discussed; that is the presence of a high intrinsic uncouling of the redox pumps (or of a fraction of completely uncoupled redox pumps). One can visualize that the existence of a fraction of the electrons being transferred without proton pumping is somehow equivalent, with respect to the effects on the P/O ratio and the RCR, to the existence of a subpopulation of uncoupled vesicles transferring electrons at maximal rate. Simulations with our model show that at high affinity of the redox reaction the patterns of the P/O ratio and the RCR as a function of f_e and f_p are indeed similar when the uncoupling is assumed to be due either to a fraction of leaky vesicles or to intrinsic uncoupling of the proton pumps. However, they become different when the affinity of the redox reaction is sufficiently lowered. Part of the difference is due to the fact that the value of $\Delta\tilde{\mu}_H$ in the stationary state of phosphorylation and therefore (due to the non linear flow-force relations) the value of the conductances of the two pumps, are different in the presence of the two different mechanisms of uncoupling. This complicates the analysis and makes the simulated patterns too much model-dependent. We have thus preferred to concentrate our analysis on the case of the inhomogeneity. One should, however, keep in mind that a high intrinsic uncoupling of the redox proton pumps can be an alternative or an additional explanation for the loose coupling of SMP's.

inability of simulating the experimental finding of a flux control equal to 1 for both redox and ATPase pumps does not critically depend on the value of the leak conductance in the population of coupled vesicles. In fact, even assuming a much higher leak conductance, in the simulations at low affinity where the redox pumps have a higher flux control with respect to the ATPases, the flux control of the ATPases remains always lower than 1 (and that of the redox pumps becomes higher than 1).

In these simulations the ratio between the conductances of the ATPase and redox pumps, $r = L_p/L_e$, at the $\Delta\tilde{\mu}_H$ of the stationary state of phosphorylation is close to 1. We have shown in the Theory section that the presence of a high leak is more likely to account for the finding of proportional titration curves with both an inhibitor of the primary and of the secondary proton pumps if the ratio between the conductances of the ATPase and redox pumps, $r = L_p/L_e$, is low. The value of r which one can derive from the relations between rate of ATP synthesis and $\Delta\tilde{\mu}_H$ and rate of electron transfer and $\Delta\tilde{\mu}_H$ as measured in mitochondria upon inhibition of the redox and the ATPase pumps respectively, is close to 1 at high affinity of the redox reaction [26]. The value of r should then be higher than 1 with the low concentration of durohydroquinone at which the experiments in Fig. 9 have been performed.

However, in view of the uncertainties on the experimental value of r (deriving mainly from the fact that the flow-force relations have been observed to depend on how $\Delta\tilde{\mu}_H$ was varied) we have investigated whether different and in particular lower values of L_p/L_e in the model could allow the simulation of the experimental titrations of Fig. 9. For each value of r (obtained by varying in the model the number of ATPase pumps with respect to that of redox pumps) the 'coupling parameters' (L_H^{nl} and f_{SMP}) have been found which allows to fit the experimental patterns of the P/O ratio and of the RCR as a function of the fraction of active pumps and the experimental values of P/O ratio and the RCR at low and high affinity. Then the rate of ATP synthesis as a function of f_e and f_p in the presence of the same combination of leak and uncoupled vesicles has been computed.

The result of this modeling study is that it is impossible to find a value of L_p/L_e and a combination of coupling parameters able to simulate all the observations and in particular the titrations of Fig. 9. The dotted curves in Figs. 9–11 (which are obtained with a fraction 0.13 of uncoupled vesicles and the same L_H^{nl} as in curves 2) are the simulations closest to the experimental behaviour which could be obtained with a different r (0.4). Even assuming the most unfavourable case that the low P/O ratio and RCR were exclusively due to an increased leak conductance in the entire population of vesicles, it has been impossible to find a value of L_p/L_e which allows to simulate the finding of an inhibition of the rate of ATP synthesis proportional to both the fraction of inhibited redox and ATPase pumps. The key fact to understand this is that as L_p/L_e decreases (whether because L_p is lower or L_e is higher) the value of the leak conductance which gives the same low (0.6) value of P/O becomes lower and lower with respect to the conductance of the redox pumps. As a result, at very low values of L_p/L_e such that the flux control of the ATPases is equal to 1 (relative rate of ATP synthesis equal to f_p in the ATPase inhibitor titrations), the flux control of the redox pumps is lower than 1 (relative rate of ATP synthesis higher than f_e in the redox inhibitor titrations). On the other hand, at (higher) values of L_p/L_e such that the flux control of the redox pumps is 1 the flux control of the ATPases is lower than 1. It can then be concluded that even in the presence of lower values of the ratio between the conductances of the two proton pumps, r, a flux control coefficient equal to 1 for both the redox and ATPase proton pumps cannot be simulated with the chemiosmotic model of energy coupling.

Taking into account the results as a whole together with the simulations and analysis presented in this paper the general conclusion emerges that a completely delocalized mechanism of free-energy coupling cannot account for the results of inhibitor titrations in submitochondrial particles. We would like to stress that, at variance from the double-inhibitor titrations approach previously used [5–7,9], the conclusions based on the single-inhibitor titrations of the present study are valid for any type of proton pump kinetics.

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