Klingenberg, M. (1964) in *Biological Oxidation* (Singer, T. P., Ed.) p 3, Wiley, New York.

Kroger, A., & Klingenberg, M. (1973a) *Eur. J. Biochem. 34*, 358–368.

Kroger, A., & Klingenberg, M. (1973b) *Eur. J. Biochem. 39*, 313–323.

Lenaz, G., & DeSantis, A. (1985) in *Coenzyme Q* (Lenaz, G., Ed.) pp 165–199, Wiley, New York.

Leung, K. H., & Hinkle, P. C. (1975) *J. Biol. Chem. 250*, 8467–8471.

Lowry, O. H., Rosebrough, N. J., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Ludwig, B., Downer, N. W., & Capaldi, R. A. (1979) *Biochemistry 18*, 1402–1407.

Mendel-Hartvig, I., & Nelson, B. D. (1978) *FEBS Lett. 92*, 36–40.

Millett, F., Darley-Usmar, V., & Capaldi, R. A. (1982) *Biochemistry 21*, 3857–3862.

Mitchell, P. (1968) *Chemiosmotic Coupling and Energy Transduction*, Glynn Research Ltd, Bodmin.

Poore, V. M., Fitzsimmon, J. T. R., & Ragan, C. I. (1982) *Biochim. Biophys. Acta 693*, 113–124.

Quintanilha, A., Thomas, D. D., & Swanson, M. (1982) *Biophys. J. 37*, 68–69.

Racker, E. (1972) *J. Membr. Biol. 10*, 221–235.

Ragan, C. I., & Cottingham, I. R. (1985) *Biochim. Biophys. Acta 811*, 13–31.

Reiske, J. S., Hansen, R. E., & Zaugg, W. S. (1962) *J. Biol. Chem. 237*, 1681–1685.

Schneider, H., Lemasters, J. J., Hoechli, M., & Hackenbrock, C. R. (1980) *J. Biol. Chem. 255*, 3748–3756.

Schneider, H., Lemasters, J. J., & Hackenbrock, C. R. (1982) *J. Biol. Chem. 257*, 10789–10793.

Sjostrand, F. G., & Cassel, R. Z. (1978) *J. Ultrastruct. Res. 63*, 111–137.

Smith, R. J., Capaldi, R. A., Muchmore, D., & Dahlguish, F. (1978) *Biochemistry 17*, 3719–3723.

Takemori, S., & King, T. E. (1964) *J. Biol. Chem. 239*, 3546–3557.

Thomas, D. D., Dalton, L. R., & Hyde, J. S. (1976) *J. Chem. Phys. 65*, 3006–3024.

Trumpower, B. L., & Katki, A. (1979) in *Membrane Proteins in Energy Transduction* (Capaldi, R. A., Ed.) pp 90–200, Marcel Dekker, New York.

Tsong, T. Y., & Knox, B. E. (1984) *Biophys. J. 45*, 296a.

Wainio, W. W. (1983) *Biol. Rev. 58*, 131–156.

Wikstrom, M., Krab, K., & Saraste, M. (1981) *Annu. Rev. Biochem. 50*, 623–655.

Wilkinson, D. A., & Nagle, J. H. (1981) *Biochemistry 20*, 187–192.

Yu, C. A., & Yu, L. (1980) *Biochim. Biophys. Acta 591*, 409–420.

Yu, C. A., Gwak, S.-H., & Yu, L. (1985) *Biochim. Biophys. Acta 812*, 656–664.

Yu, L., & Yu, C. A. (1982) *J. Biol. Chem. 257*, 2016–2021.

Ziegler, D. M., & Doeg, K. A. (1962) *Arch. Biochem. Biophys. 97*, 41–50.

Double-Inhibitor and Uncoupler–Inhibitor Titrations. 1. Analysis with a Linear Model of Chemiosmotic Energy Coupling

Daniela Pietrobon*,‡,§ and S. Roy Caplan‡

Department of Membrane Research, The Weizmann Institute of Science, Rehovot 76100, Israel, and CNR Unit for the Study of the Physiology of Mitochondria and Institute of General Pathology, University of Padova, 35100 Padova, Italy

Received December 11, 1985; Revised Manuscript Received July 15, 1986

ABSTRACT: The results of double-inhibitor and uncoupler–inhibitor titrations have been simulated and analyzed with a linear model of delocalized protonic coupling using linear nonequilibrium thermodynamics. A detailed analysis of the changes of the intermediate $\Delta\tilde{\mu}_H$ induced by different combinations of inhibitors of the proton pumps has been performed. It is shown that with linear flow–force relationships the published experimental results of uncoupler–inhibitor titrations are not necessarily inconsistent with, and those of double-inhibitor titrations are inconsistent with, a delocalized chemiosmotic model of energy coupling in the presence of a negligible leak. Also shown and discussed are how the results are affected by a nonnegligible leak and to what extent the shape of the titration curves can be used to discriminate between localized and delocalized mechanisms of energy coupling.

In the chemiosmotic view (Mitchell, 1966) energy transduction by oxidative phosphorylation in mitochondria and bacteria, as well as by photophosphorylation in chloroplasts, is accomplished by redox ($\Delta\tilde{\mu}_H$[1] generating, primary) and ATPase ($\Delta\tilde{\mu}_H$ utilizing, secondary) proton pumps coupled through the common intermediate $\Delta\tilde{\mu}_H$, which is the thermodynamically and kinetically competent driving force for ATP synthesis. Despite much supporting evidence for and wide acceptance of this delocalized protonic coupling mechanism, a series of reports in the past few years has raised doubts as to whether it provides a completely correct description of biological energy transduction (for reviews see: Westerhoff et al., 1984; Ferguson, 1985). Many of the re-

---

‡The Weizmann Institute of Science.

§University of Padova.

[1] Abbreviations: $\Delta\tilde{\mu}_H$, transmembrane difference in the electrochemical potential of protons; ATPase, adenosinetriphosphatase; ATP, adenosine triphosphate.

0006-2960/86/0425-7682$01.50/0

ported observations have been taken to indicate a localized protonic coupling mechanism in which pathways of proton flow are situated within or adjacent to the membrane and/or a direct interaction takes place between redox and ATPase pumps. This could occur either in parallel to a delocalized chemiosmotic mechanism (Rottenberg et al., 1967; Rottenberg, 1978; Boyer, 1984; Slater et al., 1985) or as the sole coupling mechanism (Williams, 1961; Kell, 1979; Westerhoff et al., 1984).

As a means of discriminating between delocalized and localized mechanisms of energy coupling, great importance has been attributed to the approach using double-inhibitor titrations described originally by Baum and co-workers (Baum et al., 1971) and more recently by Kell and co-workers (Hitchens & Kell, 1982; Westerhoff et al., 1984), who also introduced, as an extension of the method, the uncoupler–inhibitor titrations (Hitchens & Kell, 1983). In double-inhibitor titrations in different systems and with different combinations of primary and secondary pumps, it has been found that the relative inhibition of the $\Delta\tilde{\mu}_H$-driven reaction by a given concentration of inhibitor of one type of pump (e.g., redox) was the same whether the other type of pump (e.g., ATPase) was partially inhibited or not (Baum et al., 1971; Hitchens & Kell, 1982; Parsonage & Ferguson, 1982; Venturoli & Melandri, 1982). One case has been reported in which the relative inhibition by an inhibitor of the secondary pumps was even higher when the primary pumps were partially inhibited (Baum et al., 1971; Westerhoff et al., 1983). In uncoupler–inhibitor titrations it has been found that the relative inhibition of the rate of the energy-consuming reaction by a given concentration of uncoupler was greater in the presence of an inhibitor of the secondary pumps than in its absence (Hitchens & Kell, 1983; Berden et al., 1984).

The results of both types of titration have been considered as unequivocally inconsistent with delocalized models of energy coupling (Baum et al., 1971; Hitchens & Kell, 1982, 1983; Venturoli & Melandri, 1982; Berden et al., 1984; Westerhoff et al., 1984). It has been stressed that they are independent of the type of relation holding between rates (of electron transfer and of ATP synthesis) and $\Delta\tilde{\mu}_H$ and therefore of the measurements of $\Delta\tilde{\mu}_H$ (Hitchens & Kell, 1982, 1983). This conclusion was reached largely on intuitive grounds. A more quantitative analysis of the double-inhibitor titrations within a delocalized chemiosmotic model with linear flow–force relationships has been performed by Westerhoff and co-workers using control theory (Westerhoff et al., 1983; Westerhoff, 1983). They concluded that in the presence of a nonnegligible leak the application of the "rule of thumb", whereby the extent to which one of the pumps controls the flux should be decreased when the other pump is partially inhibited, becomes dubious. Moreover, they warned, predictions based on such a rule cannot be made unequivocally when dependence of reaction rates on $\Delta\tilde{\mu}_H$ varies between the different points in the titration experiment, i.e., with nonlinear flow–force relationships. And indeed very recently Davenport, using a simple nonlinear model of delocalized chemiosmotic coupling in chloroplasts, obtained simulated titration curves similar to those found experimentally (Davenport, 1985). Mills, using an analogous (but slightly more complicated) model, has simulated uncoupler–inhibitor titrations and obtained results similar to those found experimentally (i.e., higher sensitivity to the uncoupler in the presence of an inhibitor of the secondary pumps than in its absence) (Mills, 1984). Pietrobon and Caplan (1985) have shown that in the case of linear flow–force relationships as well, the experimental result is predicted by a delocalized protonic coupling model, in contrast to the intuitive arguments developed elsewhere (Hitchens & Kell, 1983; Westerhoff et al., 1984; Ferguson, 1985; van der Ben & Herweijer, 1985).

In this paper the results of double-inhibitor and uncoupler–inhibitor titrations are simulated and analyzed with a linear model of delocalized protonic coupling using linear nonequilibrium thermodynamics. Also shown and discussed are how the results are affected by a nonnegligible leak and to what extent the shape of the titration curves can be used to discriminate between localized and delocalized mechanisms of energy coupling. The analysis of the changes of the intermediate $\Delta\tilde{\mu}_H$ and of the flow, induced by different combinations of inhibitors of the primary and secondary pumps, performed in this study with the linear model is essential to understand the different behavior obtained with nonlinear flow–force relationships, as illustrated in the following paper.

ANALYSIS OF DOUBLE-INHIBITOR TITRATIONS

The simplest chemiosmotic protonic circuit is constituted by three elements in parallel: a $\Delta\tilde{\mu}_H$-generating (e.g., a redox) proton pump, a $\Delta\tilde{\mu}_H$-consuming (e.g., an ATPase) proton pump, and a "leak", i.e., a pathway for passive diffusion of protons through the membrane. If, for the sake of argument, the pumps are assumed to be completely coupled and the relationships between flows and forces are assumed to be proportional, the proton flow through each element can be described by the following equations (van Dam et al., 1980):

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

$$J_H^p = n_p J_p = n_p L_p (A_p + n_p \Delta\tilde{\mu}_H) \qquad (1b)$$

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

Here A_e and A_p are the positive affinities (the instantaneous negative Gibbs free energy changes) of the redox and ATP hydrolysis reactions, respectively, n_e is the H^+/e^- stoichiometry of the redox pump, n_p is the H^+/ATP stoichiometry of the ATPase pump, J_e is the rate of electron transfer, L_e is the proportionality coefficient between J_e and the driving force of the redox pump $(A_e + n_e\Delta\tilde{\mu}_H)$, J_p is the rate of ATP synthesis of hydrolysis ($J_p > 0$ indicates hydrolysis, $J_p < 0$ indicates synthesis), L_p is the proportionality coefficient between J_p and the driving force of the ATPase pump $(A_p + n_p\Delta\tilde{\mu}_H)$, J_H^l is the flow of protons through the "leak" and L_H^l is the leak conductance. $n_e^2L_e$ and $n_p^2L_p$ can be viewed as the conductances of the redox and ATPase pumps, respectively.

From eq 1a, 1b, and 1c and the condition of zero net proton flow, the values of $\Delta\tilde{\mu}_H$ and J_p in the stationary state of phosphorylation (state 3) can easily be derived as functions of the conductance of the three elements of the chemiosmotic circuit and of the stoichiometries and affinities of the reactions

$$\Delta\tilde{\mu}_H = -\frac{A_p}{n_p}\left(\frac{n_pA_e}{n_eA_p} + \frac{n_p^2L_p}{n_e^2L_e}\right) \Bigg/ \left(1 + \frac{n_p^2L_p}{n_e^2L_e} + \frac{L_H^l}{n_e^2L_e}\right) \qquad (2)$$

$$J_p = L_pA_p\left(1 - \frac{n_pA_e}{n_eA_p} + \frac{L_H^l}{n_e^2L_e}\right) \Bigg/ \left(1 + \frac{n_p^2L_p}{n_e^2L_e} + \frac{L_H^l}{n_e^2L_e}\right) \qquad (3)$$

Titrations with inhibitors of either the redox or the ATPase pump can be simulated by multiplying the conductances L_e and L_p by the inhibition factors f_e and f_p, respectively, varying from 1 (at zero inhibitor concentration) to 0 (at inhibitor concentrations that inhibit all the pumps). We initially con-

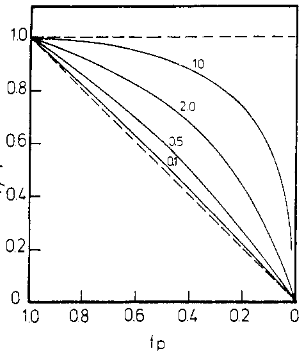

FIGURE 1: Relative or normalized rate of ATP synthesis, $J_p^p/J_p(0)$, as a function of the inhibition factor of the ATPase pumps, f_p (with $f_e = 1$). $J_p(0)$ is J_p at $f_p = 1$. The different curves refer to different values of $n_p^2L_p/(n_e^2L_e) = r$, which are indicated in the figure. The computation is based on eq 5, with $n_e = n_p = 1$. The two dashed lines correspond to the two limiting cases $r \rightarrow 0$ and $r \rightarrow \infty$. See text.

sider the situation in which $L_H^1 \ll n_e^2L_e$; i.e., the passive proton conductance is negligible in comparison with that of the redox pump (case a), and subsequently we introduce a nonnegligible leak and discuss how it modifies the behavior (case b).

(*a*) *$L_H^1 = 0$ or, in General, $L_H^1 \ll n_e^2L_e$*. To express the inhibition of the rate of ATP synthesis during the double inhibitor titrations, it is convenient to use the relative or normalized values: (a) $J_p^e/J_p(0)$ for the titration of the redox pump, where J_p^e is J_p as a function of f_e with f_p constant at a given value and $J_p(0)$ is J_p at $f_e = 1$ (zero redox inhibitor concentration) and the same constant value of f_p; (b) $J_p^p/J_p(0)$ for the titration of the ATPase pump, where J_p^p is J_p as a function of f_p with f_e constant at a given value and $J_p(0)$ is J_p at $f_p = 1$ (zero ATPase inhibitor concentration) and the same constant value of f_e. Assuming constant values for A_e and A_p during the titrations, one obtains

$$J_p^e/J_p(0) = \left(1 + f_p\frac{n_p^2L_p}{n_e^2L_e}\right) \Bigg/ \left(1 + \frac{f_p}{f_e}\,\frac{n_p^2L_p}{n_e^2L_e}\right) \qquad (4)$$

$$J_p^p/J_p(0) = f_p\left(1 + \frac{1}{f_e}\,\frac{n_p^2L_p}{n_e^2L_e}\right) \Bigg/ \left(1 + \frac{f_p}{f_e}\,\frac{n_p^2L_p}{n_e^2L_e}\right) \qquad (5)$$

The titration curve with a redox inhibitor, i.e., $J_p^e/J_p(0)$ as a function of f_e, can be calculated from eq 4 in the absence of ATPase inhibitor ($f_p = 1$) or in the presence of any arbitrary concentration of ATPase inhibitor ($f_p < 1$). Conversely, the titration curve with an ATPase inhibitor, $J_p^p/J_p(0)$ as a function of f_p, can be calculated from eq 5 in the absence of redox inhibitor ($f_e = 1$) or in the presence of any arbitrary concentration of redox inhibitor ($f_e < 1$).

Equations 4 and 5 show that for any given value of f_e and f_p the relative inhibition of the rate of ATP synthesis is uniquely determined by $n_p^2L_p/(n_e^2L_e)$, the ratio between the conductances of the two H^+ pumps in the chemiosmotic protonic circuit. For simplicity both n_e and n_p are henceforth considered equal to 1 and we shall usually denote $n_p^2L_p/(n_e^2L_e)$ by r. Figure 1 shows the titration curves obtained with an inhibitor of the ATPase pumps (with $f_e = 1$) for different values of r indicated in the figure. The dashed lines refer to the two limiting cases (see eq 5): (1) $r \rightarrow 0$, $J_p^p/J_p(0) = f_p$, inhibition of the flow proportional to the fraction of inhibited enzymes; (2) $r \rightarrow \infty$, $J_p^p/J_p(0) = 1$, no inhibition. The titration curves obtained with an inhibitor of the redox pumps (with $f_p = 1$) are exactly inversely symmetrical to those obtained with an ATPase inhibitor. The previous values of r are now replaced by their reciprocals: if $r = 10$, the titration curve with a redox inhibitor is identical with the titration curve corresponding to $r = 0.1$ in Figure 1, and so on. With a redox inhibitor $J_p^e/J_p(0) = 1$ when $r \rightarrow 0$ and $J_p^e/J_p(0) = f_e$ when $r \rightarrow \infty$ (see eq 4).

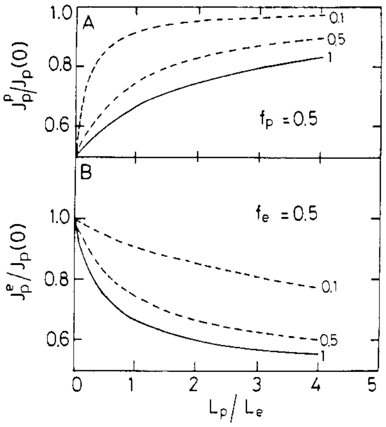

FIGURE 2: (A) Relative or normalized rate of ATP synthesis, $J_p^p/J_p(0)$, at a given concentration of ATPase inhibitor ($f_p = 0.5$), as a function of L_p/L_e ($L_p/L_e = r$ since $n_e = n_p = 1$) for different values of f_e. Continuous line: $f_e = 1$. Dashed lines: $f_e = 0.5$ and 0.1. (B) Relative or normalized rate of ATP synthesis $J_p^e/J_p(0)$ at a given concentration of redox inhibitor ($f_e = 0.5$) as a function of L_p/L_e for different values of f_p. Continuous line: $f_p = 1$. Dashed lines: $f_p = 0.5$ and 0.1. Computations from eq 4 and 5.

In the terminology of control theory (Kacser & Burns, 1973; Heinrich & Rapoport, 1974; Groen et al., 1982a,b; Westerhoff, 1983; Westerhoff et al., 1983) the limiting case $r \rightarrow 0$ corresponds to the case in which the flux control of the ATPase, ${}^PC_P$, is 1 and that of the redox pump, ${}^PC_e$, is 0 (i.e., ATPase pump completely rate limiting), while $r \rightarrow \infty$ corresponds to ${}^PC_e = 1$ and ${}^PC_P = 0$ (i.e., redox pump completely rate limiting). In the intermediate cases the flux control is distributed between the two enzymes (Westerhoff, 1983) and can be calculated for each of them from eq 3

$$\frac{\partial J_p}{\partial L_p}\frac{L_p}{J_p} \equiv {}^PC_P = \frac{1}{1 + r} \qquad (6)$$

$$\frac{\partial J_p}{\partial L_e}\frac{L_e}{J_p} \equiv {}^PC_e = \frac{r}{1 + r} \qquad (7)$$

where L_p is given by the number of ATPase pumps times the conductance of the single ATPase pump and L_e is given by the number of redox pumps times the conductance of the single redox pump. A variation in the number of ATPases or redox pumps can therefore be equivalently expressed as a variation in L_p or L_e, respectively. Note that ${}^PC_P + {}^PC_e = 1$. The flux controls of the redox and ATPase pumps, as well as the relative inhibition of J_p with either inhibitor, are uniquely dependent on the ratio r, whatever the individual values of L_p and L_e and of the affinities may be. As the ratio r increases, or in other words as the "readiness" with which the ATPases changes their rate in response to a change of $\Delta\tilde{\mu}_H$ increases with respect to that of the redox pumps, the flux control of the ATPases decreases while that of the redox pumps increases. Consequently an inhibitor of the ATPase becomes less effective in inhibiting the flow ($J_p^p/J_p(0)$ goes from f_p to 1) while an inhibitor of the redox pump becomes more effective ($J_p^e/J_p(0)$ goes from 1 to f_e). This is illustrated (cf. Figure 1) by the continuous curves in Figure 2 showing the relative inhibition of the rate of ATP synthesis by a concentration of inhibitor that eliminates half of the ATPase pumps (Figure 2A) or half of the redox pumps (Figure 2B) as a function of r.

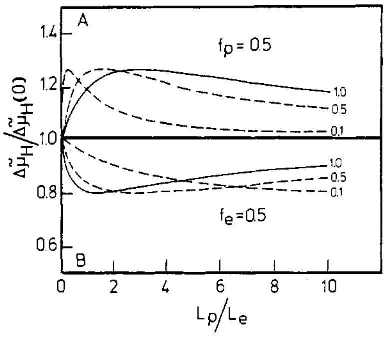

FIGURE 3: (A) Relative or normalized $\Delta\tilde{\mu}_H$ values at a given concentration of ATPase inhibitor ($f_p = 0.5$) as a function of L_p/L_e for different values of f_e. Continuous line: $f_e = 1$. Dashed lines: f_e = 0.5 and 0.1. $\Delta\tilde{\mu}_H(0)$ is $\Delta\tilde{\mu}_H$ at $f_p = 1$. (B) Relative or normalized $\Delta\tilde{\mu}_H$ values at a given concentration of redox inhibitor ($f_e = 0.5$) as a function of L_p/L_e for different values of f_p. Continuous line: f_p = 1. Dashed lines: f_p = 0.5 and 0.1. Computations from eq 1A and 2A, with $A_e = 4$, $A_p = 1$, and $n_e = n_p = 1$.

The dashed lines in Figure 2A show the relative inhibition of J_p by the same concentration of ATPase inhibitor ($f_p = 0.5$) in the presence of redox inhibitor (f_e = 0.5 or 0.1) and those in Figure 2B the relative inhibition of J_p by the same concentration of redox inhibitor ($f_e = 0.5$) in the presence of ATPase inhibitor (f_p = 0.5 or 0.1). In both cases the dashed lines are shifted upwards to higher $J_p/J_p(0)$ with respect to the continuous lines: a given inhibitor concentration (of either pump) is less effective in inhibiting the flow (J_p) when the other pump is partially inhibited. In fact a partial inhibition of the redox pump, for example, is equivalent, at any given concentration of ATPase inhibitor, to an increase of r by the factor $1/f_e$. This increase results, as discussed above, in a higher flux control of the redox pumps and a lower inhibition by the ATPase inhibitor (cf. eq 4–7). In Figure 2A $J_p^p/J_p(0)$ with $f_e = 0.5$ at each values of r is equal to that at $2r$ with $f_e = 1$. Conversely a partial inhibition of the ATPase pump is equivalent, at any given concentration of redox inhibitor, to a decrease of r by the factor f_p, resulting in a lower flux control by the redox pumps and a lower inhibition by the redox inhibitor.

In a linear chemiosmotic model of energy coupling with a negligible leak the rule of thumb whereby in the presence of a delocalized intermediate the extent to which one of the enzymes controls the flux should be decreased when the other enzyme is inhibited is always verified.

Inhibition of either pump is accompanied by changes in the magnitude of the delocalized intermediate $\Delta\tilde{\mu}_H$. When the redox pumps are titrated, $|\Delta\tilde{\mu}_H|$ (in state 3) progressively decreases since the positive $\Delta\tilde{\mu}_H$ generating proton efflux is progressively inhibited. On the other hand, when the ATPase pumps are titrated, $|\Delta\tilde{\mu}_H|$ (in state 3) progressively increases toward its static head value, since the $\Delta\tilde{\mu}_H$ dissipating proton influx is progressively decreased.

For any given value of f_e and f_p and of the ratio between the two affinities A_e and A_p, the relative or normalized values of $\Delta\tilde{\mu}_H$ are uniquely determined by r (cf. eq 1A and 2A in the Appendix). Figure 3 shows that $\Delta\tilde{\mu}_H^e/\Delta\tilde{\mu}_H(0)$ as a function of r goes through a minimum, while $\Delta\tilde{\mu}_H^p/\Delta\tilde{\mu}_H(0)$ goes through a maximum. At any given inhibition of the redox pump (in the example shown in Figure 3B, $f_e = 0.5$), the presence of an inhibitor of the ATPase pump (f_p = 0.5 and 0.1 in the dashed lines of Figure 3B) brings about a shift of the minimum to higher values of r (cf. eq 3A). Conversely at any given inhibition of the ATPase pump (f_p = 0.5 in Figure 3A), the presence of a redox inhibitor (f_e = 0.5 and 0.1 in the dashed lines of Figure 3A) brings about a shift of the maximum to lower values or r (cf. eq 5A). Note, however, that at any given inhibition of one kind of pump, the values of $\Delta\tilde{\mu}_H^e/\Delta\tilde{\mu}_H(0)$ at the minimum or $\Delta\tilde{\mu}_H^p/\Delta\tilde{\mu}_H(0)$ at the maximum are both independent of the partial inhibition of the other pump (cf. eq 4A and 6A in the Appendix).

The consequence of this behavior is that in a double-inhibitor titration the relative decrease in $|\Delta\tilde{\mu}_H|$ induced by a certain concentration of redox inhibitor can be higher or lower in the presence of an ATPase inhibitor, depending on the value of r. The relative inhibition of the rate of ATP synthesis by different combinations of inhibitors therefore does not simply correlate with the relative changes in $\Delta\tilde{\mu}_H$: a higher relative decrease of $|\Delta\tilde{\mu}_H|$ (a lower $\Delta\tilde{\mu}_H^e/\Delta\tilde{\mu}_H(0)$) can be accompanied by a lower relative inhibition of J_p (a higher $J_p^e/J_p(0)$), (cf. Figure 2 and 3). This rather nonintuitive result derives from the fact that $J_p/J_p(0)$ depends not only on $\Delta\tilde{\mu}_H/\Delta\tilde{\mu}_H(0)$ but also on $\Delta\tilde{\mu}_H(0)$. Thus from eq 1b

$$\frac{J_p^e}{J_p(0)} = \left(\frac{A_p}{n_p\Delta\tilde{\mu}_H(0)} + \frac{\Delta\tilde{\mu}_H^e}{\Delta\tilde{\mu}_H(0)}\right) \Bigg/ \left(\frac{A_p}{n_p\Delta\tilde{\mu}_H(0)} + 1\right) \tag{8}$$

$\Delta\tilde{\mu}_H(0)$ is itself dependent on r and varies as a function of r from $\Delta\tilde{\mu}_H(0) = -A_e/n_e$ when $r \rightarrow 0$ to $\Delta\tilde{\mu}_H(0) = -A_p/n_p$ when $r \rightarrow \infty$, i.e., from the static head value of the redox pump to the static head value of the ATPase pump.

(*b*) *General Case with Leak.* In both kinds of titrations a leak in parallel determines a higher inhibition of the rate of ATP synthesis by a given concentration of inhibitor (see eq 7A and 8A in the Appendix). A leak tends to compress all the titration curves of Figure 1 toward the limiting case $J_p^p/J_p(0) = f_p$; i.e., in the presence of a high leak the ATPase can be titrated almost proportionally even if the value of r is relatively high. The titration curves obtained with an inhibitor of the redox pumps are no longer symmetrical (in the sense described above) to those obtained with an inhibitor of the ATPases. Hyperbolical titration curves can also be obtained ($J_p^e/J_p(0) < f_e$). Note that especially at low r (<1) a leak could easily give rise to proportional inhibition for both kind of pumps, i.e., to a flux control of 1 for both pumps. In control theory a leak has a negative flux control and it increases the flux control of both pumps (see eq 13A and 14A in the Appendix and cf. Westerhoff, 1983). As discussed by Westerhoff (1983) and Westerhoff et al. (1983) the situation ${}^PC_P = 1$, ${}^PC_e = 1$ is reached when ${}^PC_l = -1$.

The higher inhibition of the rate of ATP synthesis by a given concentration of inhibitor in the presence of a leak is due to the fact that the leak determines a lower $\Delta\tilde{\mu}_H^e/\Delta\tilde{\mu}_H(0)$ for any given f_e, i.e., a higher relative decrement of $|\Delta\tilde{\mu}_H|$ during a redox titration, and also a lower $\Delta\tilde{\mu}_H^p/\Delta\tilde{\mu}_H(0)$ for any given f_p, i.e., a lower relative increment of $|\Delta\tilde{\mu}_H|$ during an ATPase titration. It turns out that the normalized value of the rate of ATP synthesis in the presence of a leak relative to that in the absence of leak, $(J_p/J_p(0))^l/(J_p/J_p(0))$, is *equal* to the normalized value of $\Delta\tilde{\mu}_H$ in the presence of a leak relative to that in its absence, $(\Delta\tilde{\mu}_H/\Delta\tilde{\mu}_H(0))^l/(\Delta\tilde{\mu}_H/\Delta\tilde{\mu}_H(0))$, during an ATPase titration, while it is *proportional* to it during a redox titration (see eq 9A–12A in the Appendix).

Figure 4A shows $(\Delta\tilde{\mu}_H^p/\Delta\tilde{\mu}_H(0))^l/(\Delta\tilde{\mu}_H^p/\Delta\tilde{\mu}_H(0))$ as a function of r for $f_p = 0.5$ and $f_e = 1$ (continuous line) or f_e = 0.5 and 0.1 (dashed lines). The ratio r is varied by varying L_e with L_p kept constant; as r increases, $L_H^l/n_e^2L_e$ also increases and $\Delta\tilde{\mu}_H^p/\Delta\tilde{\mu}_H(0)$ becomes progressively lower in comparison to its value at zero leak. At any given concentration of ATPase inhibitor a partial inhibition of the redox pump is equivalent

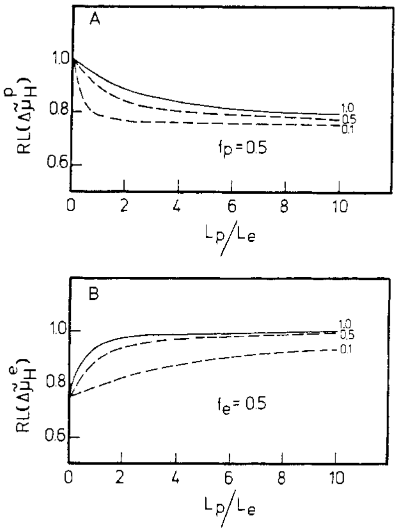

FIGURE 4: (A) Normalized values of $\Delta\tilde{\mu}_H$ at a given concentration of ATPase inhibitor ($f_p = 0.5$) in the presence of a leak relative to those in the absence of the leak, $RL(\Delta\tilde{\mu}_H{}^p) = (\Delta\tilde{\mu}_H{}^p/\Delta\tilde{\mu}_H(0))^l/(\Delta\tilde{\mu}_H{}^p/\Delta_H(0))$, as a function of L_p/L_e for different values of f_e. L_p/L_e is varied by varying L_e. Continuous line: $f_e = 1$. Dashed lines: $f_e = 0.5$ and 0.1. Computation from eq 10A and 12A, with $L_H{}^l/n_p{}^2L_p = 0.5$ and $n_e = n_p = 1$. (B) Normalized values of $\Delta\tilde{\mu}_H$ at a given concentration of redox inhibitor ($f_e = 0.5$) in the presence of a leak relative to those in the absence of the leak, $RL(\Delta\tilde{\mu}_H{}^e) = (\Delta\tilde{\mu}_H{}^e/\Delta\tilde{\mu}_H(0))^l/(\Delta\tilde{\mu}_H{}^e/\Delta\tilde{\mu}_H(0))$, as a function of L_p/L_e for different values of f_p. L_p/L_e is varied by varying L_p. Continuous line: $f_p = 1$. Dashed lines: $f_p = 0.5$ and 0.1. Computation from eq 9A and 11A, with $A_e = 4$, $A_p = 1$, $n_e = n_p = 1$, and $L_H{}^l/n_e{}^2L_e = 0.5$.

to an increase of r by the factor $1/f_e$ and also to an increase of $L_H{}^l/n_e{}^2L_e$ by the same factor. The consequence is that the same leak determines a larger decrease in $\Delta\tilde{\mu}_H{}^p/\Delta\tilde{\mu}_H(0)$ and therefore in $J_p{}^p/J_p(0)$ when the redox pumps are partially inhibited than when they are not (the dashed lines in Figure 4A are all below the continuous line). The differential effect of the leak on the ATPase titration curves in the presence or absence of a redox inhibitor reduces and tends to compensate the difference in the titration curves predicted by the rule of thumb. Indeed a sufficiently high leak leads to identical titration curves by the ATPase inhibitor in the presence or absence of redox inhibitor.

Figure 4B shows that in the other type of double-inhibitor titration also the same leak determines a larger decrease in $\Delta\tilde{\mu}_H{}^e/\Delta\tilde{\mu}_H(0)$ and therefore in $J_p{}^e/J_p(0)$ when the ATPases are partially inhibited than when they are not. This differential effect can again lead to identical titration curves.

ANALYSIS OF UNCOUPLER–INHIBITOR TITRATIONS

In the linear model of chemiosmotic energy coupling discussed earlier (see eq 1a, 1b, and 1c) a given concentration of uncoupler (a given $L_H{}^l$) gives rise to a relative decrease in the rate of ATP synthesis $J_p{}^l/J_p(0)$, and a corresponding relative decrease in $|\Delta\tilde{\mu}_H|$, $\Delta\tilde{\mu}_H{}^l/\Delta\tilde{\mu}_H(0)$, according to the relations

$$\frac{J_p{}^l}{J_p(0)} = \left(1 - \frac{L_H{}^l}{n_e{}^2L_e\left(\dfrac{n_pA_e}{n_eA_p} - 1\right)}\right) \Bigg/ \left(1 + \frac{L_H{}^l}{n_e{}^2L_e\left(1 + f_p\dfrac{n_p{}^2L_p}{n_e{}^2L_e}\right)}\right) \tag{9}$$

$$\frac{\Delta\tilde{\mu}_H{}^l}{\Delta\tilde{\mu}_H(0)} = 1 \Bigg/ \left(1 + \frac{L_H{}^l}{n_e{}^2L_e\left(1 + f_p\dfrac{n_p{}^2L_p}{n_e{}^2L_e}\right)}\right) \tag{10}$$

where $J_p{}^l$ and $\Delta\tilde{\mu}_H{}^l$ are J_p and $\Delta\tilde{\mu}_H$, respectively, as functions of $L_H{}^l$ with f_p constant at a given value and $f_e = 1$, and $J_p(0)$ and $\Delta\tilde{\mu}_H(0)$ refer to the rate of ATP synthesis and $\Delta\tilde{\mu}_H$ with $L_H{}^l = 0$ and the same constant value of f_p. Equations 9 and 10 show that at any given $L_H{}^l$ (and any given ratio of affinities) the relative decrease in the rate of ATP synthesis is proportional to the relative decrease in $|\Delta\tilde{\mu}_H|$. A computation of $J_p{}^l/J_p(0)$ as a function of $L_H{}^l$ at different f_p's shows that the relative inhibition of the rate of ATP synthesis by a given concentration of uncoupler is always higher ($J_p{}^l/J_p(0)$ is lower) in the presence of ATPase inhibitor than in its absence (Pietrobon & Caplan, 1985). As discussed in Pietrobon and Caplan (1985), this result arises from the fact that inhibition of the ATPases is accompanied by an increase in the state 3 $|\Delta\tilde{\mu}_H|$, and therefore the same concentration of uncoupler leads in the presence of inhibitor to a higher dissipative H^+ flow. Consequently, there is a higher relative decrement in $|\Delta\tilde{\mu}_H|$ accompanied by a relatively larger inhibition of J_p, since $J_p{}^l/J_p(0)$ is proportional to $\Delta\tilde{\mu}_H{}^l/\Delta\tilde{\mu}_H(0)$.

DISCUSSION

We show in this study that *linear* flow–force relationships (between the rate of electron transfer and $\Delta\tilde{\mu}_H$ and between the rate of ATP synthesis and $\Delta\tilde{\mu}_H$) within a delocalized chemiosmotic model of energy coupling in the presence of a negligible leak, always lead, in a double-inhibitor experiment, to a lower relative inhibition of the energy-driven reaction by an inhibitor of the primary pump when the secondary pump is partially inhibited (and vice versa). Only a considerable "leak" could lead, in a linear model, to an identical or similar relative inhibition under the different conditions compared. The same conclusion was reached recently with control theory (Westerhoff et al., 1983; Westerhoff, 1983) as well as earlier by several workers on largely intuitive grounds, who supposed it to be of general validity and not only limited to linear relationships between flows and $\Delta\tilde{\mu}_H$ (e.g., Kahn, 1970; Baum et al., 1971; Hitchens & Kell, 1982). These workers therefore considered any results contradicting these expectations (in particular their own) as unequivocally incompatible with delocalized models of energy coupling. In contrast we will demonstrate in the accompanying paper that even in the presence of a negligible leak *nonlinear* flow–force relationships *can* give rise, within a delocalized chemiosmotic model of energy coupling, to similar or identical normalized titration curves when titrating with an inhibitor of the primary pump in the presence or absence of an inhibitor of the secondary pump (and vice versa) (see also: Davenport, 1985).

While linear relationships between rate of electron transfer and $\Delta\tilde{\mu}_H$ have often been measured at least in a certain range of $\Delta\tilde{\mu}_H$ (Padan & Rottenberg, 1973; Azzone et al., 1978; van Dam et al., 1980; Pietrobon et al., 1982), the measured relationships between rate of ATP synthesis and $\Delta\tilde{\mu}_H$ are usually nonlinear (e.g. Graber & Witt, 1976; Baccarini Melandri et al., 1977; Zoratti et al., 1982; Clark et al., 1983). However, even if eq 1a, 1b, and 1c may be inadequate to describe the actual dependence of the proton flow through the pumps on $\Delta\tilde{\mu}_H$, the analysis, with the linear model, of the changes of the rate of ATP synthesis and of the intermediate $\Delta\tilde{\mu}_H$, induced by different combinations of inhibitors, is important and interesting since it provides some essential information to understand, and the basis on which to discuss the different behavior introduced by nonlinearity (shown in the accompanying paper).

The key fact to understand the behavior obtained with the linear model in a double-inhibitor titration, in the presence of a negligible leak, is the exclusive dependence of the relative inhibition of the rate of ATP synthesis, by a given concentration of inhibitor, on the ratio between the conductances of the two proton pumps in the chemiosmotic protonic circuit: $n_p^2L_p/(n_e^2L_e)$ (i.e., r). As this ratio increases, or in other words, as the "readiness" with which the ATPase pumps change their rate in response to a change in $\Delta\tilde{\mu}_H$ increases with respect to that of the redox pumps, an inhibitor of the ATPases becomes less effective in inhibiting the rate of ATP synthesis while an inhibitor of the redox pumps becomes more effective. Using control theory terminology, one would say that as r increases the "flux control" of the ATPases decreases while that of the redox pumps increases (Westerhoff, 1983; and cf. eq 6 and 7). In a linear model, a concentration of inhibitor that decreases the number of active ATPases by 50% also decreases r by 50%, thus decreasing the flux control of the redox pumps and the relative inhibition by a given concentration of redox inhibitor (cf. eq 4). Note however that the presence of this concentration of ATPase inhibitor does not necessarily mean that the ATPases become rate limiting as assumed by some workers (Kahn, 1970; Baum et al., 1971; Hitchens & Kell, 1982). On the other hand, due to the effective change in r which always accompanies inhibition in a linear model, there is a redistribution of the flux control between the two pumps and that of the ATPases increases. Therefore it is not true, as argued by some workers (Clark et al., 1983; Parsonage & Ferguson, 1983; Davenport, 1985), that as long as $\Delta\tilde{\mu}_H$ is not saturating for the ATPases an invariant redox inhibitor sensitivity is expected in the presence or absence of ATPase inhibitor.

Our conclusions have been based on the analysis performed with the linear model assuming that under different conditions of inhibition the affinities of the reactions remain constant at the same value. This condition is approximately met in the experiments if initial rates of phosphorylation, upon addition of ADP, are measured.

There is another aspect of the double-inhibitor titrations which, it has been suggested, could be used to discriminate between "delocalized" and "localized" chemiosmotic mechanisms (D. B. Kell, personal communication; Davenport, 1985) and as such deserves discussion. The intuitive argument is as follows (Davenport, 1985). Unless the pumps in state 3 are working at their maximum rate (state 3 in the saturation region) the decrease in $|\Delta\tilde{\mu}_H|$ that follows partial inhibition of the redox pumps in a redox titration and the increase in $|\Delta\tilde{\mu}_H|$ that follows partial inhibition of the ATPases in an ATPase titration will allow the uninhibited pumps to work at a higher rate, thus masking the inhibition. The inhibition of the flow is expected not to be proportional to the fraction of inhibited enzymes (f_e or f_p) but to be lower.

Experimentally this is usually not found with inhibitors of the ATPase. Proportional inhibition of both the rate of uncoupled ATP hydrolysis and succinate driven ATP synthesis, parallel to the binding of [^{14}C]DCCD has been reported in mitochondria (Kopecky et al., 1984). In other systems binding experiments were not performed, but an inhibition of the flow proportional to the fraction of inhibited ATPases was inferred from the parallel inhibition of the rate of ATP synthesis and uncoupled ATP hydrolysis (Ferguson & John, 1978; Venturoli & Melandri, 1982; Berden et al., 1984). A known exception of this kind of behavior is the inhibition of mitochondrial ATPases with oligomycin (Zoratti et al., 1982). If this is considered a peculiarity of that inhibitor (or a reflection of an high flux control by the ADP–ATP translocator (Lemasters & Sowers, 1979; Groen et al., 1982a,b; Zoratti et al., 1982; cf. however, Kopecky et al., 1984)) and if the possibility can be excluded that state 3 is in the saturation region of the curve J_p vs. $\Delta\tilde{\mu}_H$, proportional inhibition of the rate of ATP synthesis might be considered evidence for a localized mode of energy coupling (given that the intuitive argument developed above is correct). Against $\Delta\tilde{\mu}_H$ being saturating for the rate of ATP synthesis are the measured relations between rate of ATP synthesis and $\Delta\tilde{\mu}_H$ and the hyperbolical shape of the titrations of the rate of ATP synthesis with uncoupler (Hitchens & Kell, 1983; Berden et al., 1984). Figure 8 of the accompanying paper shows that if state 3 is in the saturation region or close to it (case B), a sigmoidal titration curve is expected.

However, a clear-cut conclusion in favor of local coupling based on the finding of an inhibition of the rate of ATP synthesis proportional to the fraction of inhibited ATPases is not possible since the intuitive argument on which it is based is only partly correct. First of all, in the presence of a negligible leak, the relative inhibition of the rate of ATP synthesis is simply related to the ratio between the "readiness" with which the two proton pumps react to a change in $\Delta\tilde{\mu}_H$, r, and not to the relative changes in $\Delta\tilde{\mu}_H$ following inhibition (which are themselves dependent on that ratio). Figure 1 shows that when the ratio of "readiness" is lower than 1 (which means the flux control of the ATPases higher than that of the redox pumps) the inhibition of the rate of ATP synthesis by an ATPase inhibitor is not proportional to the fraction f_p of inhibited enzymes but very close to it, so close that the difference could escape experimental detectability. In this case however the relative inhibition of the rate of ATP synthesis by a redox inhibitor should be substantially lower than the fraction of inhibited redox pumps.

In summary, a clear-cut conclusion based on the shape of the titration curve with ATPase inhibitors is not possible unless it can be shown either that r in state 3 is sufficiently high or that the titration curve with a redox inhibitor is not sigmoidal as would be expected with a low r. Sigmoidal titration curves have been found with redox inhibitors in mitochondria (Zoratti et al., 1982; Stoner, 1985) but not in chromatophores (Hitchens & Kell, 1982; Kell et al., 1978). In all these studies the rate of ATP synthesis has been measured as a function of the total concentration of added inhibitor and not as a function of the fraction of inhibited redox pumps, which is the relevant parameter. The results of the only work we know in which the relevant parameters can be derived, although in a rather indirect way, seem to indicate an inhibition of the rate of ATP synthesis proportional to both the fractions of inhibited redox and ATPase pumps (Venturoli & Melandri, 1982). This result is in conflict with delocalized chemiosmosis if it can be shown that the leak in chromatophores is sufficiently small (see below).

The discussion until this point has been developed by assuming a negligible leak pathway. From our analysis it is clear that if the membranes are sufficiently leaky the results of double-inhibitor titrations and also the proportional inhibition of the rate of ATP synthesis by an ATPase inhibitor are more easily accommodated within a delocalized chemiosmotic mechanism. In fact, in the presence of a high leak, proportional inhibition of the flow with inhibitors of both kind of pumps is possible, within a delocalized chemiosmotic model, especially if r is low, and, even with a relative high value of this ratio, inhibition of the flow proportional to the fraction of inhibited ATPases can be found. This is due to the effect of the leak on the relative changes of $\Delta\tilde{\mu}_H$ in state 3 following inhibition of either pump: it determines a higher relative decrease of $|\Delta\tilde{\mu}_H|$ by a given concentration of redox inhibitor and a lower relative increase of $|\Delta\tilde{\mu}_H|$ by a given concentration of an ATPase inhibitor, with a consequent larger relative inhibition of the rate of ATP synthesis with respect to the case with a negligible leak. On the other hand, in a double-inhibitor titration, the differential effect of the leak on the relative changes of $\Delta\tilde{\mu}_H$ induced by a given concentration of redox inhibitor in the presence or absence of an ATPase inhibitor (or vice versa) can lead to similar or identical inhibition of the flow in the two conditions.

A direct knowledge of the magnitude of the leak is therefore necessary if one wants to discriminate unequivocally between delocalized and localized mechanisms of energy coupling on the basis of the results of inhibitor titrations. In particular what is relevant is the magnitude of the passive conductance of the membrane, $L_H{}^1$, with respect to the conductance of the redox pumps, $n_e{}^2L_e$. In our opinion, in view of the well-known loose coupling of the submitochondrial particle and chromatophore preparations with which all the inhibitor titrations have been performed (as indicated by low P/O and respiratory control ratios) a direct measure of the above mentioned parameters is necessary. The fact that J_p/J_e in state 3 does not significantly change upon extensive inhibition of the rate of electron transfer (Clark et al., 1983; V. Petronilli et al., unpublished experiments) is not a sufficient criterion to exclude a significant leak if the passive ionic conductance depends sharply on $\Delta\tilde{\mu}_H$ and/or there is a subpopulation of uncoupled or broken vesicles (or a significant slip in the proton pumps) contributing to the loose coupling. The first point is made clear by the results of Clark et al. (1983), who show that in chromatophores most of the dissipative ionic current is through the ATPases (either deprived of F_1 or intrinsically uncoupled) and is therefore sharply dependent on $\Delta\tilde{\mu}_H$, thus accounting for the measured constancy of the P/O ratio in an extensive range of inhibition. The second point is made clear by simulations performed with the model illustrated in the accompanying paper, showing that in the presence of a subpopulation of uncoupled particles and/or intrinsic uncoupling of the redox pumps J_p/J_e would increase upon inhibition of the rate of electron transfer (Pietrobon, 1986). Preliminary results obtained in our laboratory indicate that a subpopulation of uncoupled or broken vesicles indeed contributes to the loose coupling of submitochondrial particles (V. Petronilli et al., unpublished experiments).

In the case of the uncoupler–inhibitor titrations we show that a delocalized chemiosmotic model with linear flow–force relationships gives rise to the experimentally found higher effectiveness of an uncoupler in the presence of ATPase inhibitor. The role played in this behavior by the increase in $|\Delta\tilde{\mu}_H|$ that follows inhibition of the ATPases and the consequent increase in dissipative proton flow at any given concentration of uncoupler are amply discussed elsewhere (Pietrobon & Caplan, 1985).

ACKNOWLEDGMENTS

We thank Prof. G. F. Azzone for constant support and helpful discussions.

APPENDIX

With $L_H{}^1 = 0$ (or $L_H{}^1 \ll n_e{}^2L_e$) the relative or normalized value of $\Delta\tilde{\mu}_H$ in state 3, in a titration with an inhibitor of the redox pumps $\Delta\tilde{\mu}_H{}^e/\Delta\tilde{\mu}_H(0)$, is (from eq 2)

$$\Delta\tilde{\mu}_H{}^e/\Delta\tilde{\mu}_H(0) = \left(\frac{n_pA_e}{n_eA_p} + \frac{f_p}{f_e}r\right)(1 + f_pr)\bigg/\left(\frac{n_pA_e}{n_eA_p} + f_pr\right)\left(1 + \frac{f_p}{f_e}r\right) \tag{1A}$$

where $\Delta\tilde{\mu}_H(0)$ is $\Delta\tilde{\mu}_H$ in the absence of redox inhibitor (f_e = 1) and $r = n_p{}^2L_p/(n_e{}^2L_e)$. The relative or normalized value of $\Delta\tilde{\mu}_H$ in state 3 in a titration with an inhibitor of the ATPases, $\Delta\tilde{\mu}_H{}^p/\Delta\tilde{\mu}_H(0)$, is

$$\Delta\tilde{\mu}_H{}^p/\Delta\tilde{\mu}_H(0) = \left(\frac{n_pA_e}{n_eA_p} + \frac{f_p}{f_e}r\right)\left(1 + \frac{r}{f_e}\right)\bigg/\left(\frac{n_pA_e}{n_eA_p} + \frac{r}{f_e}\right)\left(1 + \frac{f_p}{f_e}r\right) \tag{2A}$$

where $\Delta\tilde{\mu}_H(0)$ is $\Delta\tilde{\mu}_H$ in the absence of ATPase inhibitor (f_p = 1). The value of r at which $\Delta\tilde{\mu}_H{}^e/\Delta\tilde{\mu}_H(0)$ is minimal is given by

$$r_{\min}{}^e = \frac{1}{f_p}\left(f_e\frac{n_pA_e}{n_eA_p}\right)^{1/2} \tag{3A}$$

and the corresponding value of $\Delta\tilde{\mu}_H{}^e/\Delta\tilde{\mu}_H(0)$ is

$$(\Delta\tilde{\mu}_H{}^e/\Delta\tilde{\mu}_H(0))_{\min} = \left(\left(f_e\frac{n_pA_e}{n_eA_p}\right)^{1/2} + 1\right)^2\bigg/\left(\left(\frac{n_pA_e}{n_eA_p}\right)^{1/2} + f_e{}^{1/2}\right)^2 \tag{4A}$$

while the value of r at which $\Delta\tilde{\mu}_H{}^p/\Delta\tilde{\mu}_H(0)$ is maximum and the corresponding value of $\Delta\tilde{\mu}_H{}^p/\Delta\tilde{\mu}_H(0)$ are

$$r_{\max}{}^p = f_e\left(\frac{1}{f_p}\,\frac{n_pA_e}{n_eA_p}\right)^{1/2} \tag{5A}$$

$$(\Delta\tilde{\mu}_H{}^p/\Delta\tilde{\mu}_H(0))_{\max} = \left(f_p{}^{1/2} + \left(\frac{n_pA_e}{n_eA_p}\right)^{1/2}\right)^2\bigg/\left(\left(f_p\frac{n_pA_e}{n_eA_p}\right)^{1/2} + 1\right)^2 \tag{6A}$$

In the general case with leak the relative or normalized values of the rate of ATP synthesis in titrations with redox and ATPase inhibitors are (from eq 3)

$$J_p{}^e/J_p(0) = \left(1 + f_pr + \frac{L_H{}^1}{n_e{}^2L_e}\right)\left(1 - \frac{L_H{}^1}{f_en_e{}^2L_e\left(\frac{n_pA_e}{n_eA_p} - 1\right)}\right)\bigg/\left(1 + \frac{f_p}{f_e}r + \frac{L_H{}^1}{f_en_e{}^2L_e}\right)\left(1 - \frac{L_H{}^1}{n_e{}^2L_e\left(\frac{n_pA_e}{n_eA_p} - 1\right)}\right) \tag{7A}$$

$$J_p{}^p/J_p(0) = f_p\left(1 + \frac{r}{f_e} + \frac{L_H{}^1}{f_en_e{}^2L_e}\right)\bigg/\left(1 + \frac{f_p}{f_e}r + \frac{L_H{}^1}{f_en_e{}^2L_e}\right) \tag{8A}$$

The normalized values of the rate of ATP synthesis in the presence of leak (eq 7A and 8A) relative to those in the absence of leak (eq 4 and 5) are

$$\left(\frac{J_p{}^e}{J_p(0)}\right)^1 \Bigg/ \left(\frac{J_p{}^e}{J_p(0)}\right) = \left(1 + \frac{L_H{}^1}{n_e{}^2L_e}\,\frac{1}{(1+f_pr)}\right) \times \left(1 - \frac{L_H{}^1}{f_en_e{}^2L_e\left(\dfrac{n_pA_e}{n_eA_p} - 1\right)}\right) \Bigg/ \left(1 + \frac{L_H{}^1}{f_en_e{}^2L_e}\,\frac{1}{\left(1 + \dfrac{f_p}{f_e}r\right)}\right)\left(1 - \frac{L_H{}^1}{n_e{}^2L_e\left(\dfrac{n_pA_e}{n_eA_p} - 1\right)}\right) \quad (9A)$$

$$\left(\frac{J_p{}^p}{J_p(0)}\right)^1 \Bigg/ \left(\frac{J_p{}^p}{J_p(0)}\right) = \left(1 + \frac{L_H{}^1}{f_en_e{}^2L_e}\,\frac{1}{\left(1 + \dfrac{r}{f_e}\right)}\right) \Bigg/ \left(1 + \frac{L_H{}^1}{f_en_e{}^2L_e}\,\frac{1}{\left(1 + \dfrac{f_p}{f_e}r\right)}\right) \quad (10A)$$

Moreover, using eq 2, one obtains

$$\left(\frac{J_p{}^e}{J_p(0)}\right)^1 \Bigg/ \left(\frac{J_p{}^e}{J_p(0)}\right) = \left[\left(\frac{\Delta\tilde{\mu}_H{}^e}{\Delta\tilde{\mu}_H(0)}\right)^1 \Bigg/ \left(\frac{\Delta\tilde{\mu}_H{}^e}{\Delta\tilde{\mu}_H(0)}\right)\right]\left(1 - \frac{L_H{}^1}{f_en_e{}^2L_e\left(\dfrac{n_pA_e}{n_eA_p} - 1\right)}\right) \Bigg/ \left(1 - \frac{L_H{}^1}{n_e{}^2L_e\left(\dfrac{n_pA_e}{n_eA_p} - 1\right)}\right) \quad (11A)$$

$$\left(\frac{J_p{}^p}{J_p(0)}\right)^1 \Bigg/ \left(\frac{J_p{}^p}{J_p(0)}\right) = \left(\frac{\Delta\tilde{\mu}_H{}^p}{\Delta\tilde{\mu}_H(0)}\right)^1 \Bigg/ \left(\frac{\Delta\tilde{\mu}_H{}^p}{\Delta\tilde{\mu}_H(0)}\right) \quad (12A)$$

The flux controls of the redox and ATPase pumps in the general case with leak are (from eq 3)

$$\frac{\partial J_p}{\partial L_p}\,\frac{L_p}{J_p} \equiv {}^PC_P = \left(1 + \frac{L_H{}^1}{n_e{}^2L_e}\right) \Bigg/ \left(1 + r + \frac{L_H{}^1}{n_e{}^2L_e}\right) \quad (13A)$$

$$\frac{\partial J_p}{\partial L_e}\,\frac{L_e}{J_p} \equiv {}^PC_e = -\frac{n_pA_e}{n_eA_p}r\left(1 - \frac{n_eA_p}{n_pA_e} + \frac{L_H{}^1}{n_p{}^2L_p}\right) \Bigg/ \left(1 - \frac{n_pA_e}{n_eA_p} + \frac{L_H{}^1}{n_e{}^2L_e}\right)\left(1 + r + \frac{L_H{}^1}{n_e{}^2L_e}\right) \quad (14A)$$

or

$${}^PC_e = -\frac{n_eJ_e}{n_pJ_p}r \Bigg/ \left(1 + r + \frac{L_H{}^1}{n_e{}^2L_e}\right) \quad (15A)$$

REFERENCES

Azzone, G. F., Pozzan, T., Massari, S., Bragadin, M., & Pregnolato, L. (1978) *Biochim. Biophys. Acta 501*, 296–306.

Baccarini Melandri, A. Casadio, R., & Melandri, B. A. (1977) *Eur. J. Biochem. 78*, 389–402.

Baum, H., Hall, G. S., Nalder, J., & Beechey, R. (1971) in *Energy Transduction in Respiration and Photosynthesis* (Quagliariello, E., et al., Eds.) pp 747–755, Adriatica Editrice, Bari.

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

Boyer, P. D. (1984) in *H^+-ATPase (ATP Synthase) Structure, Function, Biogenesis. The F_0–F_1 Complex of Coupling Membranes* (Papa, S., et al., Eds.) pp 329–338, ICSU Press and Adriatica Editrice, Bari.

Clark, A. J., Cotton, N. P. J., & Jackson, T. B. (1983) *Biochim. Biophys. Acta 723*, 440–453.

Davenport, J. W. (1985) *Biochim. Biophys. Acta 807*, 300–307.

Ferguson, S. J. (1985) *Biochim. Biophys. Acta 811*, 47–97.

Ferguson, S. J., & John, P. (1978) *Biochem. Soc. Trans. 5*, 1525–1527.

Gräber, P., & Witt, H. T. (1976) *Biochim. Biophys. Acta 423*, 141–163.

Groen, A. K., Van der Meer, R., Westerhoff, H. V., Wanders, R. J. A., Akerboom, J. P. M., & Tager, J. M. (1982a) in *Metabolic Compartmentation* (Siess, H., Ed.) pp 9–37, Academic, New York.

Groen, A. K., Wanders, R. J. A., Westerhoff, H. V., van der Meer, R., & Tager, J. M. (1982b) *J. Biol. Chem. 257*, 2754–2757.

Heinrich, R., & Rapoport, J. A. (1974) *Eur. J. Biochem. 42*, 89–95.

Hitchens, G. D., & Kell, D. B. (1982) *Biochem. J. 206*, 351–357.

Hitchens, G. D., & Kell, D. B. (1983) *Biochim. Biophys. Acta 723*, 308–316.

Kacser, H., & Burns, J. A. (1973) *Rate Control of Biological Processes* (Davis, D. D., Ed.) pp 65–104, Cambridge University Press, Cambridge, U.K.

Kahn, J. S. (1970) *Biochem. J. 116*, 55–60.

Kell, D. B. (1979) *Biochim. Biophys. Acta 549*, 55–99.

Kell, D. B., John, P., & Ferguson, S. J. (1978) *Biochem. Soc. Trans. 6*, 1292–1295.

Kopecký, J., Houštek, J., & Drahota, Z. (1984) in *H^+-ATPase (ATP Synthase) Structure, Function, Biogenesis. The F_0–F_1 Complex of Coupling Membranes* (Papa, S., et al., Eds.) pp 257–263, ICSU Press and Adriatica Editrice, Bari.

Lemasters, S. J., & Sowers, A. (1979) *J. Biol. Chem. 254*, 1248–1251.

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

Mitchell, P. (1966) *Chemiosmotic Coupling in Oxidative and Photosynthetic Phosphorylation*, Glynn Research Ltd., Bodmin, U.K.

Padan, E., & Rottenberg, H. (1973) *Eur. J. Biochem. 40*, 431–437.

Parsonage, D., & Ferguson, S. J. (1982) *Biochem. Soc. Trans. 10*, 257–258.

Pietrobon, D. (1986) *Bioelectrochem. Bioenerg. 15*, 193–209.

Pietrobon, D., & Caplan, S. R. (1985) *FEBS Lett. 192*, 119–122.

Pietrobon, D., Zoratti, M., Azzone, G. F., Stucki, J. W., & Walz, D. (1982) *Eur. J. Biochem. 127*, 483–494.

Rottenberg, H. (1978) *FEBS Lett. 94*, 295–297.

Rottenberg, H., Caplan, S. R., & Essig, A. (1967) *Nature (London) 216*, 610–616.

Slater, E. C., Berden, J. A., & Herweijer, M. A. (1985) *Biochim. Biophys. Acta 811*, 217–231.

Stoner, C. D. (1985) *J. Bioenerg. Biomembr. 17*, 85–108.

van Dam, K., Westerhoff, H. V., Krab, K., van der Meer, R., & Arents, J. C. (1980) *Biochim. Biophys. Acta 591*, 240–250.

van der Bend, R. L., & Herweijer, M. A. (1985) *FEBS Lett. 186*, 8–10.

Venturoli, G., & Melandri, B. A. (1982) *Biochim. Biophys. Acta 680*, 8–16.

Westerhoff, H. V. (1983) Ph.D. Thesis, University of Amsterdam, Amsterdam, The Netherlands.

Westerhoff, H. V., Colen, A. M., & van Dam, K. (1983) *Biochem. Soc. Trans. 11*, 81–85.

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

Williams, R. J. P. (1961) *J. Theor. Biol. 1*, 1–17.

Zoratti, M., Pietrobon, D., & Azzone, G. F. (1982) *Eur. J. Biochem. 126*, 443–451.

Double-Inhibitor and Uncoupler–Inhibitor Titrations. 2. Analysis with a Nonlinear Model of Chemiosmotic Energy Coupling

Daniela Pietrobon*,‡,§ and S. Roy Caplan‡

Department of Membrane Research, The Weizmann Institute of Science, Rehovot 76100, Israel, and CNR Unit for the Study of the Physiology of Mitochondria and Institute of General Pathology, University of Padova, 35100 Padova, Italy

Received December 11, 1985; Revised Manuscript Received July 15, 1986

ABSTRACT: The results of double-inhibitor and uncoupler–inhibitor titrations have been simulated and analyzed with a nonlinear model of delocalized protonic coupling obtained by linking two proton pump models of the kind studied by Pietrobon and Caplan [Pietrobon, D., & Caplan, S. R. (1985) *Biochemistry 24*, 5764–5776] through their common intermediate $\Delta\tilde{\mu}_H$. It is shown that the results predicted by a delocalized chemiosmotic model are highly dependent on the kind of relationships existing between rate of ATP synthesis, J_p, and $\Delta\tilde{\mu}_H$ and rate of electron transfer, J_e, and $\Delta\tilde{\mu}_H$. With nonlinear flow–force relationships all the results reported so far are not necessarily inconsistent with the delocalized chemiosmotic model provided that the relationships between rates and $\Delta\tilde{\mu}_H$ satisfy the following requirements: $\partial J_p/\partial\Delta\tilde{\mu}_H$ increases and/or $\partial J_e/\partial\Delta\tilde{\mu}_H$ decreases as $|\Delta\tilde{\mu}_H|$ increases.

In the preceding paper we analyzed the results of double-inhibitor and uncoupler–inhibitor titrations obtained for a delocalized chemiosmotic model of energy coupling (Mitchell, 1966) with linear flow–force relationships (between the rate of electron transfer and $\Delta\tilde{\mu}_H$,[1] and between the rate of ATP synthesis and $\Delta\tilde{\mu}_H$). However, the flow–force relationships measured in many energy-transducing systems (see references below) and also those simulated on the basis of simple kinetic models of ion pumps (Hansen et al., 1981; Chapman et al., 1983; Lauger, 1984; Pietrobon & Caplan, 1985) are approximately linear only in limited ranges of $\Delta\tilde{\mu}_H$. The measured relation between rate of electron transfer and $\Delta\tilde{\mu}_H$ obtained by decreasing $|\Delta\tilde{\mu}_H|$ below its static head value is characterized by a region of approximate linearity extending over 40–50 mV and by a saturation region of maximum electron flow at lower $|\Delta\tilde{\mu}_H|$ (Padan & Rottenberg, 1973; Nicholls, 1974; Azzone et al., 1978a; van Dam et al., 1980; Pietrobon et al., 1982). The measured relation between rate of ATP synthesis and $\Delta\tilde{\mu}_H$ is usually characterized by a threshold value of $|\Delta\tilde{\mu}_H|$ below which no ATP synthesis occurs and above which there is a sharp nonlinear dependence of the rate of ATP synthesis on $\Delta\tilde{\mu}_H$ (see for example the relations obtained by inhibiting the rate of electron transfer in mitochondria, e.g., Zoratti et al., 1982; Mandolino et al., 1983; Yagi et al., 1984; or those obtained by decreasing the light intensity in photophosphorylating systems, e.g., Graber & Witt, 1976; Baccarini Melandri et al., 1977; Hangarter & Good, 1982; Clark et al., 1983; or those obtained by varying an artificially imposed $\Delta\tilde{\mu}_H$, e.g., Graber, 1982). The general flow–force relationships for the six-state proton pump model studied by Pietrobon and Caplan (1985) are sigmoidal, a common characteristic of models of ion pumps or cotransport systems involving cyclic reaction schemes with only one voltage-dependent step (Hansen et al., 1981; Chapman et al., 1983).

The conclusion of the preceding paper that the published results of double-inhibitor titrations are inconsistent with a

---

[1] Abbreviations: $\Delta\tilde{\mu}_H$, transmembrane difference in the electrochemical potential of protons; $\Delta\psi$, transmembrane difference of electrical potential; ATPase, adenosinetriphosphatase; ATP, adenosine triphosphate; A_e, affinity of the electron-transfer reaction; A_p, affinity of the ATP hydrolysis reaction; n_p, stoichiometry H^+/ATP of the ATPase pump; n_e, stoichiometry H^+/e^- of the redox pump; J_e, rate of electron transfer; J_p, rate of ATP synthesis or hydrolysis ($J_p > 0$, hydrolysis; $J_p < 0$, synthesis); f_e, inhibition factor of the redox pumps; f_p, inhibition factor of the ATPases.

‡ The Weizmann Institute of Science.

§ University of Padova.

0006-2960/86/0425-7690$01.50/0