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MOLECULAR SLIPPING IN REDOX AND ATPase H^+ PUMPS

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The titration of the mitochondrial ATPase H^+ pump with oligomycin has been compared with the titration of the redox H^+ pump with antimycin. In both cases there is extensive inhibition of the pumps without significant depression of $\Delta\tilde{\mu}_H$. The two pumps exhibit 'nonohmic' behavior in different ranges of $\Delta\tilde{\mu}_H$. This discrepancy favors the hypothesis of nontightly coupled or 'slipping' H^+ pumps with respect to that of a steep dependence of the membrane 'leak' conductance for H^+ on $\Delta\tilde{\mu}_H$.

Although the role of the H^+ electrochemical gradient, $\Delta\tilde{\mu}_H$, as coupling intermediate is still controversial, it is generally accepted that the redox-driven H^+ pumps as well as the ATP-driven H^+ pumps create such a $\Delta\tilde{\mu}_H$ across the membrane of mitochondria, chloroplasts and bacteria [1]. Both the mitochondrial redox-driven H^+ pumps, in the presence of an electron donor and acceptor and an excess of oligomycin, and the ATP-driven H^+ pumps, in the presence of ATP and an excess of antimycin, soon reach a stationary state (resting state) called State 4 or static head.

In this state the efflux of protons, J_H^{eff}, created by the H^+ pumps is equal to the passive influx of protons, J_H^{inf}, through the so-called 'leaks':

$$J_H^{eff} = J_H^{inf} \quad (1)$$

i.e., the net H^+ flux is zero.

If a proportional relationship between J_H and $\Delta\tilde{\mu}_H$ is assumed, then:

$$J_H^{inf} = L_H^l \Delta\tilde{\mu}_H \quad (2)$$

where L_H^l is the membrane leak conductance. Furthermore, if the H^+ pumps are assumed to be tightly coupled, the two processes of electron transfer and H^+ translocation, (or of ATP hydrolysis and H^+ translocation) can be considered as stoichiometric processes. Hence:

$$J_H^{eff} = nJ_e \quad (3)$$

$$J_H^{eff} = (1+n')J_{ATP} \quad (4)$$

for the redox- and ATP-driven H^+ pumps, respectively, where $n = H^+/e^-$ and $n' = H^+/ATP$ and one additional proton originates from the electrophoretic ATP translocation [2–4].

In static head, for the redox- and ATP-driven H^+ pumps:

$$nJ_e = L_H^l \Delta\tilde{\mu}_H \quad (5)$$

$$(1+n')J_{ATP} = L_H^l \Delta\tilde{\mu}_H \quad (6)$$

Inhibition of J_e or J_{ATP} should be accompanied by a proportional decrease in $\Delta\tilde{\mu}_H$, provided that

Abbreviations: $TPMP^+$, triphenylmethylphosphonium ion; DCCD, *N,N'*-dicyclohexylcarbodiimide; DMO, 5,5-dimethyl-2,4-oxazolidinedione; EGTA, ethylenebis(oxoethylenenitrilo)-tetraacetic acid; Mops, 4-morpholinepropanesulfonic acid; J_e, rate of electron transfer; J_{ATP}, rate of ATP hydrolysis.

L^l_H, n and n' are not affected by the inhibitors per se. However, at variance from the prediction of Eqn. 5, since the pioneer experiment of Nicholls [5] it has been repeatedly reported [6–11] that J_e inhibition in static head is not accompanied by a proportional decrease in $\Delta\tilde{\mu}_H$, i.e., J_e can be substantially inhibited without significantly lowering $\Delta\tilde{\mu}_H$. The present paper reports that also Eqn. 6 does not hold: ATP hydrolysis is inhibited more than 50% without significant depression of $\Delta\tilde{\mu}_H$. Similar observations have recently been made in submitochondrial particles [12].

Two hypotheses have been proposed to explain the discrepancy. (a) Nonohmic conductance [5]: above a threshold $\Delta\tilde{\mu}_H$ value the membrane leak conductance for protons is a steep function of $\Delta\tilde{\mu}_H$: $L^l_H = f(\Delta\tilde{\mu}_H)$. (b) Not tightly coupled or slipping H^+ pumps [11]: the H^+ pumps display a certain number of 'failures' or 'slips', i.e., they may transfer electrons (hydrolyze ATP) without extruding protons and protons may reenter the matrix through the pumps without moving electrons backwards (without making ATP). Eqns. 5 and 6 are not correct because $J^{eff}_H \neq nJ_e$ and $J^{eff}_H \neq (1+n')J_{ATP}$.

This paper reports an experiment where the behavior of L^l_H vs. $\Delta\tilde{\mu}_H$, obtained from a titration with antimycin of the redox-driven H^+ pump, is compared with the behavior of L^l_H vs. $\Delta\tilde{\mu}_H$ obtained from a titration with oligomycin of the ATP-driven H^+ pump. The discrepancy between the regions of nonohmic behavior in the two cases is not compatible with hypothesis a. On the other hand, hypothesis b can completely account for the present results.

Rat liver mitochondria were prepared according to standard methods. The mitochondrial protein was assayed by the biuret method using serum albumin as a standard. The composition of the reaction medium is given in the legends to the figures. Electron flow, J_e, was estimated from the decrease in oxygen concentration of the medium which was measured polarographically with a Clark electrode, equipped with a Teflon membrane in a closed thermostattically controlled vessel with magnetic stirring. Oxygen concentration was taken to be 0.243 mM at 25°C [13]. The rate of ATP hydrolysis, J_{ATP}, was measured spectrophotometrically on an Aminco DW 2a dual-wavelength spectrophotometer equipped with magnetic stirring and thermostatic control, as the decrease in absorbance at 340 minus 374 nm due to NADH oxidation, in the presence of nonlimiting quantities of phospho*enol*pyruvate, pyruvate kinase, lactate dehydrogenase and NADH. The measured rates of ATP hydrolysis were corrected for the contribution due to extramitochondrial ATPases, which was determined in control samples in the presence of 1 μg oligomycin/mg protein and 200 μM atractyloside. The determination of $\Delta\tilde{\mu}_H$ was carried out in parallel samples on the basis of the distribution of the lipophilic cation [^{14}C]TPMP$^+$ (0.02 μCi/ml) and of the weak acid [^{14}C]DMO (0.07 μCi/ml), as described elsewhere [13]. All measurements, J_e, J_{ATP} and $\Delta\tilde{\mu}_H$, were carried out in parallel on the same mitochondrial preparation.

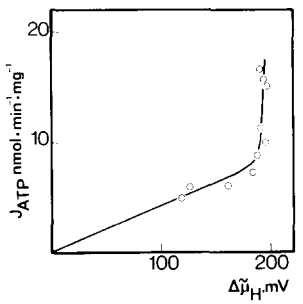

Fig. 1. Relationship between rate of ATP hydrolysis, J_{ATP}, and $\Delta\tilde{\mu}_H$ in static head for the ATP-driven H^+ pump. Titration with oligomycin. Medium composition: 0.18 M sucrose, 30 mM KCl, 10 mM Tris-Mops, 2 μM rotenone, 0.2 mM EGTA, 5 mM $MgCl_2$, 1 mM Tris-phosphate, 0.05 ng antimycin A/mg protein, 1 mM phospho*enol*pyruvate, 0.5 mM NADH and excess pyruvate kinase and lactate dehydrogenase. pH 7.4, $T = 25$°C. Mitochondria (0.5–1 mg protein/ml) were incubated for 3 min in the presence of varying concentrations of oligomycin (0–1 μg/mg protein). 2 mM ATP was then added, and after 1.5 min J_{ATP} and $\Delta\tilde{\mu}_H$ were measured.

Fig. 1 shows the relationship between rate of ATP hydrolysis, J_{ATP}, and proton electrochemical gradient, $\Delta\tilde{\mu}_H$, in static head for the ATP-driven H^+ pumps. The ATPase activity was titrated with (increasing amounts of) oligomycin, in the presence of excess antimycin to block the redox H^+ pumps. Inhibition of J_{ATP} is accompanied by a proportional decrease in $\Delta\tilde{\mu}_H$ below 180 mV; above this value J_{ATP} is inhibited more than 50% without significant depression of $\Delta\tilde{\mu}_H$.

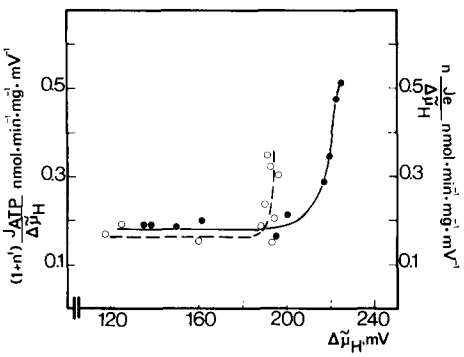

Fig. 2. Relationship between $nJ_e/\Delta\tilde{\mu}_H$ (●———●) and $\Delta\tilde{\mu}_H$ and between $(1+n')J_{ATP}/\Delta\tilde{\mu}_H$ (○———○) and $\Delta\tilde{\mu}_H$. Titration of the ATPase H^+ pump with oligomycin; medium and procedure as in Fig. 1. $n' = H^+/ATP = 3$. Titration of the redox H^+ pump with antimycin. Medium: 0.18 M sucrose, 30 mM KCl, 10 mM Tris-Mops, 2 μM rotenone, 0.2 mM EGTA, 5 mM $MgCl_2$, 2 mM ATP, 1.5 μg oligomycin/mg protein, pH 7.4. $T = 25°C$. ATP and $MgCl_2$ were included to approach as closely as possible the composition of the medium used for the titration of ATPase activity. Control experiments showed that the results are not influenced by the presence in the medium of oligomycin, P_i or of the components of the ATP-regenerating system (+oligomycin). Mitochondria (2 mg protein/ml) were incubated for 4 min in the presence of varying concentrations of antimycin A (0–13 ng/mg). 5 mM Tris-succinate was then added and after 2 min J_e and $\Delta\tilde{\mu}_H$ were measured. $n = H^+/e^- = 4$.

The relationship between J_{ATP} and $\Delta\tilde{\mu}_H$ shown in Fig. 1, which resembles closely the relationship previously found between electron flow J_e and $\Delta\tilde{\mu}_H$ in analogous inhibition experiments on the redox H^+ pumps in static head, is at variance from the prediction of Eqn. 6.

Both hypotheses a and b can explain the result shown in Fig. 1, but this is not the case with the experiment of Fig. 2. Fig. 2 compares the relationship between $nJ_e/\Delta\tilde{\mu}_H$ and $\Delta\tilde{\mu}_H$ (i.e., according to eqn. 5, L^l_H vs. $\Delta\tilde{\mu}_H$), during a titration of the redox H^+ pump with antimycin, with the relationship between $(1+n')\,J_{ATP}/\Delta\tilde{\mu}_H$ and $\Delta\tilde{\mu}_H$ (i.e., according to Eqn. 6, L^l_H vs. $\Delta\tilde{\mu}_H$) during a titration of the ATPase H^+ pump with oligomycin. Fig. 2 allows one to discriminate between the two hypotheses. If hypothesis a holds, the two functions $nJ_e/\Delta\tilde{\mu}_H$ and $(1+n')J_{ATP}/\Delta\tilde{\mu}_H$ plotted against $\Delta\tilde{\mu}_H$ should give exactly the same curve, because they are both equal to $L^l_H = f(\Delta\tilde{\mu}_H)$. In contrast, Fig. 2 shows that the threshold $\Delta\tilde{\mu}_H$ value, above which there is a very steep rise of the apparent membrane conductance L^l_H, is different for the two pumps. This indicates that $nJ_e/\Delta\tilde{\mu}_H$ and $(1+n')J_{ATP}/\Delta\tilde{\mu}_H$ are not equivalent to the same function $L^l_H = f(\Delta\tilde{\mu}_H)$. Only in the region where the apparent conductance is constant (ohmic region) do their values become similar (0.17–0.18 $nmol \cdot min^{-1} \cdot mg^{-1} \cdot mV^{-1}$). The difference between the two functions in Fig. 2 is, on the other hand, not surprising if hypothesis b holds. In this case, the nonvanishing electron flow (ATP hydrolysis) at static head is due not only to a leak conductance for protons, but also to molecular slipping in the H^+ pumps. Within this concept, the relative constancy of $\Delta\tilde{\mu}_H$ during the titrations is a consequence of the simultaneous inhibition of both the pumping and slipping processes. The two functions $nJ_e/\Delta\tilde{\mu}_H$ and $(1+n')J_{ATP}/\Delta\tilde{\mu}_H$ do not represent L^l_H, a property of the lipid bilayer, but rather, or also, the characteristics of the two slipping redox and ATPase H^+ pumps, which are presumably different.

It is possible to express rigorously $J_e/\Delta\tilde{\mu}_H$ and $J_{ATP}/\Delta\tilde{\mu}_H$ in terms of the relevant kinetic and thermodynamic parameters of the two different H^+ pumps, by applying the formalism of nonequilibrium thermodynamics.

In terms of linear nonequilibrium thermodynamics, a nontightly coupled or slipping redox H^+ pump, obeying symmetrical flow-force relationship, is described by [13] (see Refs. 3 and 4 for a nonequilibrium thermodynamics treatment of tightly coupled H^+ pumps):

$$J_e = L_e A_e + L_{eH}\Delta\tilde{\mu}_H \tag{7a}$$

$$J_H = L_{eH}A_e + L_H\Delta\tilde{\mu}_H + L^l_H\Delta\tilde{\mu}_H \tag{7b}$$

where A_e is the affinity of the electron-transfer reaction (equal to $-\Delta G_x/2$, and the L coefficients are generalized phenomenological conductances. By convention, $J_H(\text{in} \rightarrow \text{out}) > 0$ and therefore $\Delta\tilde{\mu}_H = \tilde{\mu}^{in}_H - \tilde{\mu}^{out}_H$. Two important parameters can be defined [14]: (i) the intrinsic degree of coupling of the H^+ pump $q \equiv L_{eH}/\sqrt{L_e L^P_H}$ and (ii) the phenomenological stoichiometry $Z = \sqrt{L^P_H/L_e}$ (Z is equal to n, the mechanistic stoichiometry, only when $q = 1$, but for a nontightly coupled pump $q < 1$). By introducing these two parameters into Eqns. 7a and 7b with the condition $J^{sh}_H = 0$, the

following equation for the static head of redox-driven H^+ pump can be derived [15]:

$$J_e = -\left[L_e(c)\,Z\left(\frac{1}{q} - q\right) + \frac{L_H^l}{qZ}\right]\Delta\tilde{\mu}_H \qquad (8)$$

where $L_e(c)$ is the phenomenological conductance of the respiratory chain as a function of the concentration of inhibitor (c). $L_e(c)$ then decreases with the increase in inhibitor concentration. Eqn. 8 is reduced to Eqn. 5 for $q = 1$, i.e., for tightly coupled H^+ pumps. However, if $q < 1$, the function $J_e/\Delta\tilde{\mu}_H$ is given by the sum of two terms: one, L_H^l/qZ, is a measure of the leak membrane conductance L_H^l, and the other, $L_e(c)\,Z(1/q - q)$, which increases as q decreases, is a measure of the extent of intrinsic uncoupling of the H^+ pumps, i.e., of slips. The latter term reflects an intrinsic property of the pump and is therefore likely to differ for the redox and ATPase H^+ pumps. For a given q, $J_e/\Delta\tilde{\mu}_H$ is a function of inhibitor concentration and hence it should decrease with the increase in pump inhibition. In fact, as Fig. 3 shows, the patterns of the two functions, $J_e/\Delta\tilde{\mu}_H$ vs. antimycin concentration and $J_{ATP}/\Delta\tilde{\mu}_H$ vs. oligomycin concentration, resemble closely the inhibition curve of J_e and J_{ATP}, respectively. In the course of the titration the relative contribution of leaks and slips changes in favor of the former. Only at high inhibitor concentration, when the contribution of slips becomes negligible with respect to that of leaks, do the two functions approach the constant value L_H^l/qZ. Note that L_H^l/qZ is similar but not identical for the two H^+ pumps due to the probably different value of qZ. In the low $\Delta\tilde{\mu}_H$ region, the ratio of the slopes of the plots J_e vs. $\Delta\tilde{\mu}_H$ and J_{ATP} vs. $\Delta\tilde{\mu}_H$ is close to 1. In this region proton pumping occurs to compensate H^+ influx via leaks, which should be the same at equal $\Delta\tilde{\mu}_H$ values, while slips are abolished by the presence of the inhibitors. The result thus indicates that approximately equal numbers of protons are pumped due to hydrolysis of one ATP molecule or transfer of one electron from succinate to oxygen. For tightly coupled pumps this means that $n = n' + 1$. We have used $n = 4$, but conclusions would not be altered if n were different. At variance with the present work, Nicholls [5] has reported almost identical $\Delta\tilde{\mu}_H$ values, in static head, during respiration and ATP hydrolysis. This result would be at variance with the present observations had the experiments been carried out with the same mitochondrial preparation and under identical experimental conditions. It is not clear whether this has been the case. Note that according to our analysis, different values of the input force ΔG_{0x} and ΔG_p and/or different q/Z ratios for the two pumps are expected to lead to different values of the output force $\Delta\tilde{\mu}_H$.

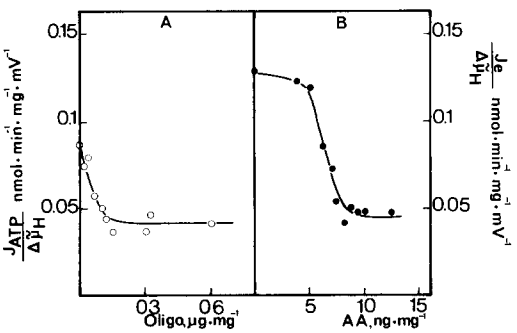

Fig. 3. Relationship between $J_{ATP}/\Delta\tilde{\mu}_H$ in static head and oligomycin concentration (A) and between $J_e/\Delta\tilde{\mu}_H$ in static head and antimycin concentration (B). Data taken from Fig. 2.

The inhibitor titrations of the mitochondrial redox H^+ pumps in the present as well as in a preceding [11] paper, can therefore be completely accounted for [15] by Eqn. 8. Evidence for linearity and symmetry in flow-force relationships and for $q < 1$, i.e., not tightly coupled redox H^+ pumps, has been recently reported [13]. Obviously, an equation analogous to Eqn. 8 for the ATP-driven H^+ pump is valid only if also this pump obeys linearity and symmetry. Studies are in progress to ascertain this point. In Ref. 12 a linear relation between net proton flux and $\Delta\tilde{\mu}_H$ has been found to hold exactly in the $\Delta\tilde{\mu}_H$ region where hypothesis a assumes nonohmicity, i.e., marked nonlinearity. Even more direct evidence for linear relationship between passive proton flux and $\Delta\tilde{\mu}_H$ has been reported by Maloney [16,17] in anaerobic, DCCD-treated bacteria. Proton influx was measured after artificial imposition of $\Delta\tilde{\mu}_H$, varied in a wide range up to more than 200 mV, across the cell membrane of the anaerobic *Streptococcus lactis*. The leak proton conductance L_H^l was constant in the whole range tested, and its value agreed well with the value obtained in 'acid pulse' experiments

where $\Delta\tilde{\mu}_H$ was close to zero. Jackson [18] has recently reported a nonlinear relationship between rate of decay and extent of carotenoid shift in bacterial chromatophores in the presence of excess oligomycin or DCCD. However, the rate of carotenoid shift decay provides the total dissipative ionic current, and not J_H. The current-voltage relationship can be different for different ions depending on the molecular mechanism of ion translocation [13,19]. The result just mentioned is therefore not necessarily in contrast with a linear relationship between J_H and $\Delta\tilde{\mu}_H$ (L^1_H = constant) [13,16,17].

Other observations, difficult to reconcile with hypothesis a of nonohmic conductance but not with hypothesis b of slipping pumps, have been reported. In inhibitor titrations of the mitochondrial redox H^+ pumps, the range of $\Delta\tilde{\mu}_H$ where $nJ_e/\Delta\tilde{\mu}_H$ (which is $L^1_H = f(\Delta\tilde{\mu}_H)$ within hypothesis a, is a function of $\Delta\tilde{\mu}_H$, is different depending on whether the electron acceptor is O_2 or ferricyanide [11]. Oxidation of succinate generates a higher $\Delta\tilde{\mu}_H$ than the oxidation of NADH although the rate of proton pumping, nJ_e, appears to be considerably lower with succinate as substrate in submitochondrial particles [10], or about the same for both substrates in mitochondria [13]. These findings indicate that $nJ_e/\Delta\tilde{\mu}_H$ is a function of the redox H^+ pump under operation as predicted by Eqn. 8.

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