Thermodynamic limits to the stoichiometry of H^+ pumping by mitochondrial cytochrome oxidase

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H^+/O stoichiometries of 0, 2 and 4 have been proposed for cytochrome oxidase. Here we show that a stoichiometry of 4 is thermodynamically impossible for rat liver cytochrome oxidase under normal conditions.

Cytochrome oxidase *Proton pumping* *H^+/O stoichiometry* *Thermodynamics* *Mitochondria*

1. INTRODUCTION

The H^+/O stoichiometry of cytochrome oxidase is still disputed. Different groups have proposed values of 0 [1,2], 2 [3,4] or 4 [5,6] (see criticism [7]), from reductant or oxygen pulse experiments at level flow (i.e. $\Delta\bar{\mu}H^+ = 0$). On going from level flow to static head (e.g. state 4), the stoichiometry might change, as pointed out by various workers [4,6], perhaps due to molecular slipping of the redox pumps [8]. This could possibly explain why stoichiometries obtained at static head by steady state methods [9] give lower values than some obtained at level flow [5,6]. However, Brand et al. [10] have observed constant stoichiometries at a range of $\Delta\bar{\mu}H^+$ values from just below state 4 to about 100 mV.

One approach to this problem is to demonstrate that a particular stoichiometry is thermodynamically impossible. This has been attempted by Wikstrom [4], using literature values of E_h for cytochrome *c* and the oxygen/water couple and a state 4 $\Delta\bar{\mu}H^+$ of 200 mV, he calculated a maximum q^+/O stoichiometry of 5.5, i.e. a maximum H^+/O stoichiometry of 3.5. However, that work was inconclusive as it used data from different sources not obtained under comparable conditions.

Here we show that, if the relevant $\Delta\bar{\mu}H^+$ is delocalized, then a H^+/O stoichiometry of 4 is thermodynamically impossible in state 4, and only becomes feasible at $\Delta\bar{\mu}H^+$ values below about 150 mV.

Abbreviations: H^+/O, number of protons pumped from the matrix to the external phase per pair of electrons passed through cytochrome oxidase; q^+/O, net number of positive charges translocated outwards across the inner mitochondrial membrane per pair of electrons passed through cytochrome oxidase; $\Delta\bar{\mu}H^+$, external proton electrochemical potential – intramitochondrial proton electrochemical potential; $\Delta\psi$, external electrical potential – intramitochondrial electrical potential; ΔpH, external pH – intramitochondrial pH; J_o, rate of oxygen consumption; E_m, midpoint potential; E_h, actual redox potential; ΔG, Gibbs free energy difference; TPMP, methyltriphenylphosphonium; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine

2. EXPERIMENTAL

2.1. *Theory*

The reaction occurring at cytochrome oxidase, where 2 electrons are passed through the membrane, *n* protons are pumped from the matrix (M) to the external phase (E), and 2 protons are taken up from the matrix by the reduction of oxygen, is thermodynamically equivalent [11] to the reaction:

$$2\text{cyt } c^{2+} + (n+2)H^+(M) + 1/2O_2(E) \rightleftharpoons 2\text{cyt } c^{3+} + H_2O(E) + nH^+(E)$$

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where the free energy change (expressed in mV) is given by

$$-\Delta G/F = 2\Delta E_h - (n + 2)[\Delta\psi - 59\Delta pH]$$

For a reaction to be possible, the thermodynamic driving force, $-\Delta G/F$, must be greater than 0.

2.2. *Materials and methods*

Rat liver mitochondria were prepared after [14], in 250 mM sucrose, 5 mM Tris and 1 mM EGTA, pH 7.4, and assayed for protein concentration after [15], using bovine serum albumin as a standard. Mitochondrial matrix volume was estimated using [^{14}C]sucrose (0.1 μCi/ml) as an extramitochondrial matrix marker, and ^3H_2O (1 μCi/ml) for total pellet volume. Matrix volume was determined as water space − sucrose space. ΔpH was determined by [^{3}H]acetate (1 μCi/ml) accumulation, with [^{14}C]sucrose as an extramitochondrial marker, ΔpH = −59log(acetate accumulation ratio), where the acetate accumulation ratio = [(acetate space − sucrose space)/ matrix volume] (see [12]). $\Delta\psi$ was measured using a TPMP-sensitive membrane electrode [16], with a correction for binding of 0.33 [12]. Hence, $\Delta\psi$ = 59log(0.33 × TPMP accumulation ratio). The electrode was calibrated with small additions of TPMP. The response was linear with log[TPMP], down to 0.5 μM TPMP. Oxidation rates were estimated polarographically using a Clark electrode, calibrated assuming 474 nmol O/ml at 25°C [17]. Percentage reduction of cytochrome *c* was determined using a Perkin-Elmer 557 dual beam spectrophotometer, using the wavelength pair 550–540 nm. Zero percent reduction was taken before the addition of substrate in aerobic and rotenone containing medium. 100% reduction was taken after addition of potassium cyanide. Subsequent addition of a small amount of dithionite caused no further reduction. When using ascorbate/TMPD as a substrate, a correction was made for a small initial increase in absorption on adding ascorbate, since control experiments showed it not to be due to cytochrome *c* reduction. When using succinate as substrate, exogenous cytochrome *c* was added to facilitate observation [15]. This procedure gave identical results to those obtained using only endogenous cytochrome *c*. It was not possible to do this when using ascorbate/TMPD as substrate because direct reduction of exogenous cytochrome *c* forced it away from redox equilibrium with the endogenous cytochrome *c* pool.

The redox potential for the O_2/H_2O couple was calculated using the external pH. This gave the same result as using the internal pH for the oxygen reaction and correcting for ΔpH. The midpoint potential of cytochrome *c* was taken as 245 mV [12]. Taking the midpoint potential of the $\frac{1}{4}O_2/\frac{1}{2}H_2O$ couple as 820 mV at pH 7.0 [13], the E_h for this couple in 50% air saturated medium is 805 mV, and it changes from 809 to 795 mV on going from 90 to 10% saturated medium. All experiments were carried out in this range of oxygen saturation. Hence the redox drop (in mV) across cytochrome oxidase at 50% air saturation is given by:

$$\Delta E_h = 805 - [245 + RT/F\ln\{(100 - x)/x\}]$$

(x = % reduction of cytochrome *c*)

The experimental protocol used was to incubate mitochondria (2 mg protein/ml) in the following medium: 120 mM KCl, 10 mM K-Hepes, 5 mM mannitol, 1 mM K-EGTA, 1 mM $MgCl_2$, 10 μM K-acetate, 5 μM $TPMP^+Br^-$, 8.3 μM rotenone, 0.1 μg/ml nigericin at 25°C and pH 7.0. Other constituents of the media are given in the figure legends. $\Delta\psi$ and J_o were determined simultaneously, the experimental points were obtained successively whilst substrates or uncoupler were titrated in over 2–5 min. Matrix volume, ΔpH and % reduction were measured in parallel on separate samples. For the radioactive assays, each experimental value was obtained after a 3 min incubation of the mitochondria. The % reduction was measured for each experimental point, 0% reduction and 100% reduction were measured before and after the determination. The sample was left 2–3 min after addition of substrate to come to a stable value. All points were the average of 3 determinations, and in all cases the standard errors were less than the size of the points shown.

3. RESULTS

The approach used was to measure $\Delta\psi$, ΔpH and ΔE_h, and then to calculate the thermodynamic driving force for the 3 stoichiometries of 0, 2 and

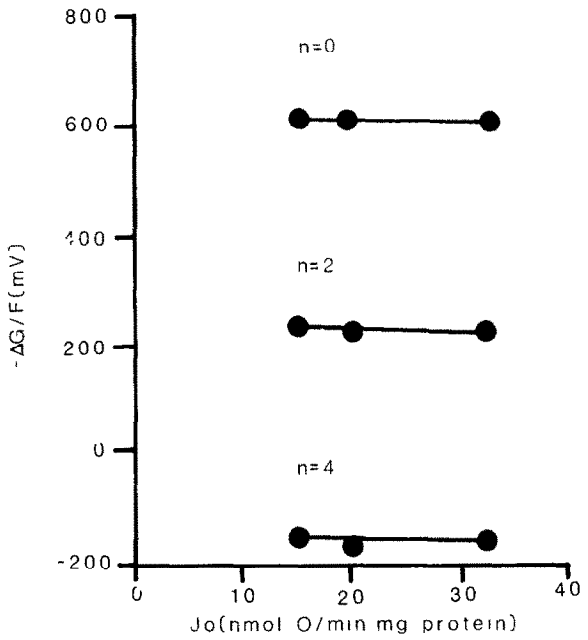

Fig.1. Thermodynamics of the cytochrome oxidase reaction at different assumed H^+ stoichiometries: State 4–state 3 transition. The thermodynamic force across cytochrome oxidase was calculated for the three stoichiometries 0, 2 and 4. The steady states were for the state 3–state 4 transition and were achieved, in order of increasing J_o, by succinate; succinate and an initial pulse of ADP which was allowed to equilibrate; succinate, ADP and hexokinase, maintaining a constant ADP concentration. The medium used was as in section 2 with the following additions: 5 mM K_2HPO_4, 5 mM glucose, 1.3 μM cytochrome *c*, 8.3 mM K-succinate, 0.156 mM ADP and 3.3 μg/ml hexokinase. ΔpH was consistently about 10 mV, % reduction of cytochrome *c* varied around 20–22% and $\Delta\psi$ was in the range 177–188 mV.

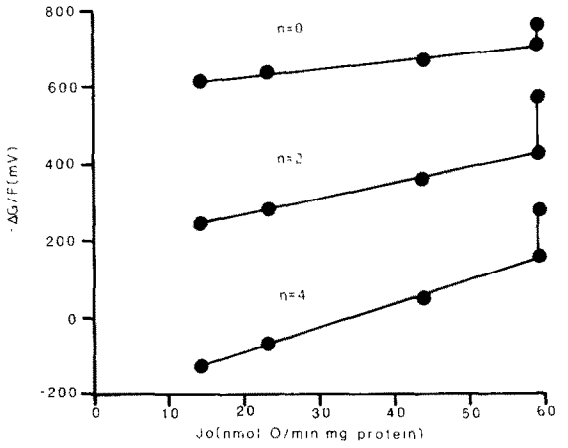

Fig.2. Thermodynamics of the cytochrome oxidase reaction at different assumed H^+ stoichiometries: FCCP titration. Mitochondria respiring on succinate were titrated with FCCP to lower $\Delta\bar{\mu}H^+$ and increase J_o. The thermodynamic force across cytochrome oxidase was calculated for each steady state, assuming stoichiometries of 0, 2 and 4. The line representing a stoichiometry of 4 crosses the $-\Delta G/F = 0$ line at a $\Delta\bar{\mu}H^+$ of about 150 mV. The medium used is described in section 2 with the following additions: 8.3 mM K-succinate, 1.3 μM cytochrome *c* and 0.3 μg/ml oligomycin. FCCP was titrated in the range 0–1.16 μM. ΔpH was about 10 mV for all the experiments, % reduction of cytochrome *c* varied from 15 to 20% and $\Delta\psi$ dropped from 180 to about 100 mV.

4. These values are shown plotted against J_o in figs 1–4. Fig.1 shows the thermodynamic driving force between state 3 and state 4 in the presence of nigericin. Clearly, under these conditions $n = 4$ is an impossible stoichiometry. The low value of J_o in state 3 was always seen in the presence of nigericin, however, experiments in nigericin-free media (not shown) confirmed our conclusion for state 4, but allowed the possibility of $n = 4$ below a state 3 $\Delta\bar{\mu}H^+$ of around 150 mV, which agrees with the experiment shown in fig.2.

When J_o was raised and $\Delta\bar{\mu}H^+$ lowered by titration with the uncoupler FCCP as shown in fig.2, $n = 4$ only became thermodynamically feasible when J_o was above about 35–40 nmol O/min per mg protein. This corresponded to a $\Delta\bar{\mu}H^+$ of below about 150 mV. In fig.3 electron transport was driven using ascorbate as substrate, with various amounts of TMPD added. This experiment again shows that it is impossible for cytochrome oxidase to pump 4 H^+ per O except when $\Delta\bar{\mu}H^+$ is low, as it is during the early stages of the titration.

In fig.4 $\Delta\bar{\mu}H^+$ was clamped at a high value with a high concentration of exogenous ATP and then TMPD was titrated in to raise J_o. This high value of $\Delta\bar{\mu}H^+$ rendered $n = 4$ impossible throughout the TMPD titration.

Similar results were obtained for an FCCP titration of mitochondria where cytochrome *c* was held more oxidized by partial inhibition with malonate while respiring on succinate. Experiments on mitoplasts, respiring on succinate or ascorbate/TMPD also gave results similar to those shown above for mitochondria.

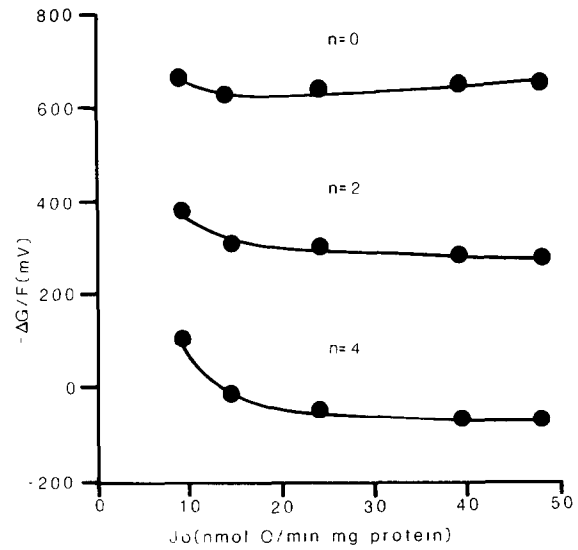

Fig.3. Thermodynamics of the cytochrome oxidase reaction at different assumed H^+ stoichiometries: TMPD titration. $\Delta\bar{\mu}H^+$ and J_o were increased by titrating in TMPD and the thermodynamic force across cytochrome oxidase was calculated assuming stoichiometries of 0, 2 and 4. The medium used was as in section 2 with the following additions: 0.76 μg/ml oligomycin, 0.18 μM antimycin and 7.36 mM K-ascorbate. TMPD was titrated in the concentration range 0–200 μM. The % reduction of cytochrome *c* varied from 0 to 30% and $\Delta\psi$ increased from 100 to 180 mV during the experiment while ΔpH stayed in the range 0–10 mV.

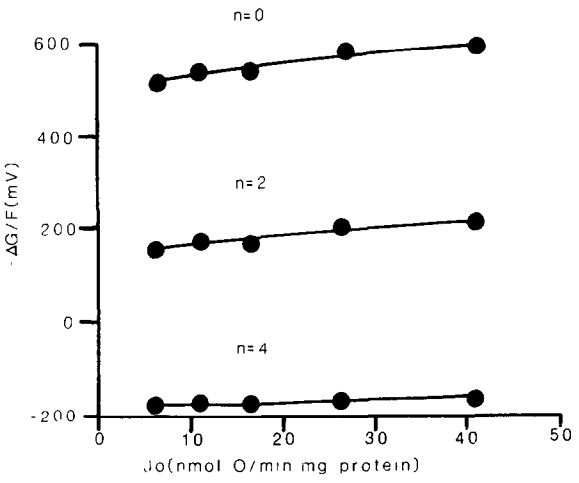

Fig.4. Thermodynamics of the cytochrome oxidase reaction at different assumed H^+ stoichiometries: TMPD titration at high fixed $\Delta\bar{\mu}H^+$. J_o was titrated with TMPD while $\Delta\bar{\mu}H^+$ was held at a high value by a large concentration of exogenous ATP. The thermodynamic force across cytochrome oxidase was calculated for stoichiometries of 0, 2 and 4. For medium used see section 2 with the following additions: 0.27 μM antimycin, 2.8 mM ATP and 7.36 mM K-ascorbate. TMPD was titrated in the range 0–200 μM. % reduction of cytochrome *c* varied from 0 to 30% and $\Delta\psi$ increased slightly on adding TMPD from 165 to 180 mV. ΔpH was in the range 10–20 mV.

4. DISCUSSION

This work is the first experimental test of the thermodynamic limits on proton pumping by cytochrome oxidase. It shows $n = 4$ to be an impossible stoichiometry in state 4 and confirms and extends the earlier theoretical treatment by Wikstrom [4]. Our results do not exclude a mechanistic stoichiometry of 4 at level flow, where $\Delta\bar{\mu}H^+$ is below 150 mV. However, as the stoichiometry appears to be invariant between state 4 and $\Delta\bar{\mu}H^+$ values down to about 100 mV [10] it seems unlikely that $n = 4$ occurs in energized mitochondria, even when it is thermodynamically possible.

The above approach assumes that the measured $\Delta\bar{\mu}H^+$ is the thermodynamic force felt by cytochrome oxidase. This would not be the case if localised proton circuits existed [18]. Localised effects could potentially invalidate the state 3 experimental points shown in fig.1 but in the uncoupler titration of fig.2 local and delocalized pools of protons would probably have equilibrated and our analysis would still be correct. Hence, even if localised pools of protons exist $n = 4$ would still be impossible for $\Delta\bar{\mu}H^+$ values above 150 mV.

An additional assumption is that the protons for the oxygen reaction are taken up from the matrix. Were these protons to be taken up from the external phase our results would eliminate the possibility of pumping 6 protons across the inner mitochondrial membrane per oxygen atom in state 4.

The conclusion we draw from these experiments is that cytochrome oxidase cannot pump 4 protons per O as its normal role. It could in theory do so when $\Delta\bar{\mu}H^+$ is below about 150 mV. It is not possible on the strength of these experiments to decide

between a stoichiometry of 0 or 2. However, as much evidence has now accumulated to show that cytochrome oxidase does pump protons [4], it seems most probable that, in vivo, it operates as a proton pump, pumping 2 H^+ and translocating 2 further charges for each oxygen atom reduced.

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REFERENCES

[1] Moyle, J. and Mitchell, P. (1978) FEBS Lett. 88, 268–272.
[2] Papa, S., Capuano, F., Markert, M. and Altamura, N. (1980) FEBS Lett. 111, 243–248.
[3] Wikstrom, M.K. (1977) Nature 266, 271–273.
[4] Wikstrom, M.K., Krab, K. and Saraste, M. (1981) Annu. Rev. Biochem. 50, 623–655.
[5] Azzone, G.F., Pozzan, T. and DiVirgilio, F. (1979) J. Biol. Chem. 254, 10206–10212.
[6] Costa, L., Reynafarje, B. and Lehninger, A.L. (1984) J. Biol. Chem. 259, 4802–4811.
[7] Krab, K., Soos, J. and Wikstrom, M.K. (1984) FEBS Lett. 178, 187–192.
[8] Pietrobon, D., Azzone, G.F. and Walz, D. (1981) Eur. J. Biochem. 117, 389–394.
[9] Al-Shawi, M.K. and Brand, M.D. (1981) Biochem. J. 200, 539–546.
[10] Brand, M.D., Harper, W.G., Nicholls, D.G. and Ingledew, W.J. (1978) FEBS Lett. 95, 125–129.
[11] Ferguson, S.J. and Sorgato, M.C. (1982) Annu. Rev. Biochem. 51, 185–217.
[12] Brown, G.C. and Brand, M.D. (1985) Biochem. J. 225, 399–405.
[13] George, P. (1965) in: Oxidases and Related Redox Systems (King, T. ed.) vol.1, pp.3–33, Wiley, New York.
[14] Chappell, J.B. and Hansford, R.G. (1972) in: Subcellular Components: Preparation and Fractionation (Birnie, G. ed.) 2nd edn, pp.77–91, Butterworths, London.
[15] Gornall, A.G., Bardawill, C.J. and David, M.A. (1948) J. Biol. Chem. 177, 751–766.
[16] Kamo, N., Muratsugu, M., Hongoh, R. and Kobatake, Y. (1979) J. Membrane Biol. 49, 105–121.
[17] Chappell, J.B. (1964) Biochem. J. 19, 225–237.
[18] Westerhoff, H.V., Melandri, B.A., Venturoli, G., Azzone, G.F. and Kell, D.B. (1984) Biochim. Biophys. Acta 768, 257–292.