Thermodynamic control of electron flux through mitochondrial cytochrome *bc*₁ complex

Guy C. BROWN and Martin D. BRAND

Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CB2 1QW, U.K.

(Received 12 July 1984/Accepted 2 October 1984)

1. The redox states of exogenously added ubiquinone-2 and cytochrome *c*, and the protonmotive force (Δp) of rat liver mitochondria were measured as the respiration rate was titrated with the uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazine. 2. The force ratio $\Delta E_h/\Delta p$ across the *bc*₁ complex was close to 1:1 in State 4, indicating an H^+/e^- stoichiometry of 1:1 for the cytochrome *bc*₁ complex, excluding protons moved by pool ubiquinone, 3. Assuming a constant stoichiometry the rate of electron transport increased linearly with the disequilibrium ($\Delta E_h - \Delta p$) across the complex.

The chemiosmotic theory (Mitchell, 1966) gives a framework within which to describe mitochondrial respiratory control. The theory predicts that the rate of flow of electrons down the mitochondrial respiratory chain is a function of the redox state of respiratory intermediates (E_h) and the protonmotive force (Δp) across the mitochondrial inner membrane. This is due to the compulsory coupling of electron flow through the respiratory complexes to the ejection of protons across the membrane.

From the Second Law of Thermodynamics we can deduce that at equilibrium (i.e. zero rate) for a respiratory complex translocating *m* charges and *n* protons per electron against a membrane potential ($\Delta\psi$) and pH gradient (at 25°C):

$$\Delta G = -F(\Delta E_h - m\Delta\psi + n \cdot 59\Delta pH) = 0$$

where ΔG is the Gibbs free energy of the reaction and ΔE_h is the redox potential difference of electrons across the complex. Thus it is possible to estimate charge/electron and proton/electron stoichiometries by measurement of ΔE_h , $\Delta\psi$, and ΔpH

Abbreviations used: TPMP⁺, methyltriphenylphosphonium; TPP⁺, tetraphenylphosphonium; Q-2, ubiquinone-2; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazine; E_m , midpoint potential; E_h , actual redox potential; E'_h , actual redox potential at defined pH; ΔG , Gibbs free-energy difference; *F*, Faraday constant; $\Delta\psi$, electrical potential across mitochondrial inner membrane; ΔpH , pH difference across mitochondrial inner membrane; Δp , protonmotive force across mitochondrial inner membrane; J_O , rate of respiratory O₂ consumption.

across a complex, when in a steady state close to equilibrium. This approach has already been attempted for complex 1 alone (De Jonge & Westerhoff, 1982), and for complexes 1 and 3 together (Johnson & Hansford, 1977).

However, classical thermodynamics is of no help in describing the rates of reactions. Thus, although we may be tempted to suggest (or hope for) a simple relationship between ΔG and rate, as the theory of irreversible thermodynamics does (Rottenberg, 1979), there is no reason *a priori* for expecting one. It is therefore necessary to use a phenomenological description of the relationship between E_h , $\Delta\psi$, ΔpH and the rate of electron flow.

For the whole respiratory chain, several groups have shown a linear relationship between Δp and the rate of O₂ consumption (Padan & Rottenberg, 1973; Nicholls, 1974), and Rottenberg & Gutman (1977) have shown a linear relationship between substrate redox potential and the rate of electron flow. No quantification of the relationship has so far been attempted for individual complexes. This is probably due to the difficulty of measuring the redox state of ubiquinone in mitochondria, and in isolated and reconstituted complexes it is difficult to quantify Δp .

In the work presented in this paper we have measured continuously the ΔE_h between exogenously added ubiquinone (Q-2) and cytochrome *c*, and the Δp , as the respiratory rate (J_O) is titrated with the protonophore FCCP, for isolated rat liver mitochondria respiring on succinate in State 4 (nomenclature of Chance & Williams, 1955). The objectives were: (a) to estimate the proton/electron

stoichiometry of the cytochrome bc_1 complex in State 4 from the force ratio ($\Delta E_h/\Delta p$); (b) to estimate how close the complex is to equilibrium; (c) to quantify the relationship between ΔE_h , $\Delta\psi$, Δp and J_O , and test if the rate of electron flow bears a simple relationship to the ΔG of the reaction. We find that $\Delta E_h/\Delta p$ is close to 1:1 in State 4, indicating a stoichiometry of 1 proton/electron pumped vectorially by the bc_1 complex, that the ΔE_h (Q-2 to cytochrome c) is measurably larger than Δp , and that the rate of electron flow is approximately linearly related to the ΔG , up to the uncoupled rate.

Experimental

Preparation of mitochondria

Rat liver mitochondria were prepared according to standard methods (Chappell & Hansford, 1972) in a medium containing 250 mM-sucrose, 5 mM-Tris and 1 mM-EGTA, adjusted to pH 7.4 with HCl. The mitochondrial protein was assayed with the biuret method (Gornall *et al.*, 1949), with bovine serum albumin as a standard.

Measurement of matrix volume

Mitochondrial-matrix volume was measured by using either [^{14}C]mannitol (0.14 $\mu\text{Ci}/\text{ml}$, 5 mM) or [^{14}C]sucrose (0.14 $\mu\text{Ci}/\text{ml}$, 5 mM) as extramitochondrial marker (which gave identical results), and $^3\text{H}_2\text{O}$ (1.0 $\mu\text{Ci}/\text{ml}$) for total pellet volume. Matrix volume was calculated as $^3\text{H}_2\text{O}$ space – [^{14}C]mannitol space.

Measurement of Δp

Δp was calculated from the accumulation ratio of [^3H]acetate (0.8 $\mu\text{Ci}/\text{ml}$, 0.1 mM). Accumulation ratio of acetate was calculated as:

$$([\text{H}]\text{Acetate space} - [\text{H}]\text{mannitol space})/\text{matrix volume}$$

Δp was calculated as $-59 \cdot \log(\text{accumulation ratio})$.

Measurement of $\Delta\psi$

$\Delta\psi$ was measured by using the lipophilic cation TPMP $^+$, monitored either radioactively or with a TPMP $^+$ -sensitive electrode. TPMP $^+$ binds to protein and lipid, and in the matrix may have an activity of less than 1 (Holian & Wilson, 1980). The accumulation ratio of TPMP $^+$ was calibrated against the accumulation ratio of $^{86}\text{Rb}^+$, over a range of $\Delta\psi$ varied by external K^+ in the presence of excess valinomycin.

Fig. 1 shows that the relationship between TPMP $^+$ and Rb^+ accumulation ratio is linear and proportional, such that:

$$\text{Accumulation ratio for } \text{Rb}^+ = n \times \text{accumulation ratio for TPMP}^+$$

Fig. 1. Calibration of [^3H]TPMP $^+$ against $^{86}\text{Rb}^+$ uptake by rat liver mitochondria

Mitochondria (2 mg of mitochondrial protein/ml) were incubated in medium containing 250 mM-sucrose, 5 mM-Hepes (sodium salt), 5 mM-succinate (sodium salt), 1 mM-EGTA (sodium salt), 5 μM -rotenone, 0.6 μg of oligomycin/ml, 320 pmol of valinomycin/mg of protein, [^3H]TPMP $^+$ bromide (0.1 $\mu\text{Ci}/\text{ml}$, 5 μM) and 0.005 μCi of $^{86}\text{RbCl}/\text{ml}$, pH 7.00, at 25°C, for 5 min. External KCl was present from 0 to 10 mM. Matrix volume was determined in parallel by using [^{14}C]mannitol and $^3\text{H}_2\text{O}$. Average slope from two experiments was $3.03 (\pm 0.30)$, i.e. $n = 0.33$.

where n is a calibration factor for TPMP $^+$ equal to $0.33 (\pm 0.03)$, which was found to be the same in the presence of 0.2 nM or 5 μM unlabelled TPMP $^+$, and independent of the valinomycin concentration above a certain value. The hint of non-linearity at the highest potentials in Fig. 1 was not seen in other experiments, and in any case would affect the calculated potentials by less than 2 mV.

For radioactive experiments [^3H]TPMP $^+$ (0.05 $\mu\text{Ci}/\text{ml}$, 0.2 μM) was used. $\Delta\psi$ was calculated as:

$$\Delta\psi = 59 \cdot \log(0.33 \times \text{accumulation ratio for TPMP}^+)$$

Continuous monitoring of $\Delta\psi$ was achieved by using a TPMP $^+$ electrode, a modification of the TPP $^+$ electrode described by Kamo *et al.* (1979). The electrode consisted of an Ag/AgCl electrode immersed in 10 mM-TPMP $^+$ bromide, separated from the assay medium by a poly(vinyl chloride) membrane impregnated with tetraphenylboron. This was coupled with a reference electrode. The

electrodes were inserted through an air-tight port into a Clark-type oxygen-electrode vessel, fitted with magnetic stirrer, allowing continuous monitoring of $\Delta\psi$ and J_O simultaneously. The TPMP⁺-electrode response was calibrated for each incubation with 1 μ M additions of TPMP⁺ up to 5 μ M. $\Delta\psi$ was calculated as before, also by using a calibration factor for TPMP⁺ equal to 0.33.

All radioactivity experiments were done in parallel with assays of J_O and redox states, with the same media and conditions, but including the isotopically labelled compounds. Mitochondria were spun down at 12000g for 2 min, and the radioactivities of the supernatant and pellet were counted separately with a liquid-scintillation counter with quench and cross-over corrections.

Measurement of respiration rates

O₂ concentration was measured polarographically with the Clark electrode with a Teflon membrane, enclosed in a thermostatically controlled vessel.

Measurement of redox states of Q-2 and cytochrome *c*

The redox state of Q-2 was measured in a Perkin-Elmer 557 dual-beam spectrophotometer, by using the wavelength pair 290 nm–275 nm. Fully oxidized Q-2 was taken as the absorbance difference in the presence of 5 mM-malonate, and fully reduced as that in the presence of 0.5 nmol of antimycin/mg of protein or 5.0 mM-cyanide (same reduction). The $E_h(Q-2)$ was calculated as:

$$E_h = E_m' + 30 \cdot \log(\% \text{ oxidized Q-2} / \% \text{ reduced Q-2})$$

The E_m' (pH 7.0) of Q-2 was taken as +100 mV (Clark, 1960).

The redox state of cytochrome *c* (cyt. *c*) was measured by using the wavelength pair 550 nm–540 nm. Fully oxidized cytochrome *c* was taken as the absorbance difference in the presence of 5 mM-malonate and fully reduced as that in the presence of 5 mM-cyanide. $E_h(\text{cyt. } c)$ was calculated as:

$$E_h = E_0 + 59 \cdot \log(\% \text{ oxidized cyt. } c / \% \text{ reduced cyt. } c)$$

$E_0(\text{cyt. } c)$ was assessed by redox titration with dithionite, of both cytochrome *c* and NNN'-tetramethyl-*p*-phenylenediamine (TMPD) in the presence of 1 mg of mitochondrial protein/ml, 5 μ M-rotenone, 100 pmol of FCCP/mg protein, 5 mM-malonate, 0.5 nmol of antimycin/mg of protein and 5 mM-cyanide. In these conditions the E_0 of cytochrome *c* was assessed to be +245 mV (± 1.0), assuming the E_m' (TMPD) at pH 7.0 to be +240 mV (Prince *et al.*, 1981).

Experimental design

Measurements of matrix volume, $\Delta\psi$ and Δp H were carried out by using radioactivity methods, or $\Delta\psi$ by using the TPMP⁺-sensitive electrode, on the same preparation of mitochondria in parallel with assays of $E_h(Q-2)$, $E_h(\text{cyt. } c)$ and J_O . All determinations were done at least in triplicate, and each experiment was repeated on at least three separate occasions. The media used were as indicated in the Figure legends.

Materials

Q-2 was originally from Hoffman-La Roche (Basel, Switzerland), and was a gift from Dr. I. Ragan. [¹⁴C]Mannitol, [¹⁴C]sucrose, [³H]acetate, ³H₂O and ⁸⁶RbCl were from Amersham International (Amersham, Bucks., U.K.). [³H]-TPMP⁺ bromide was from New England Nuclear Chemicals (Dreieich, Germany). All other chemicals were from Sigma Chemical Co. (Poole, Dorset, U.K.), and were of the highest quality commercially available.

Results

Effects of exogenously added Q-2 and cytochrome *c* on respiration

Addition of Q-2 (10 μ M) to 1 mg of mitochondrial protein/ml increased the uncoupled rate of respiration by about 10%. It also increased the coupled rate by 70%, but without affecting Δp . Thus it is unlikely to act by uncoupling. The effect was probably due to the stimulation of succinate dehydrogenase by ubiquinol (Gutman & Silman, 1975) or by increasing the quinone pool size. The maximum rate of reduction in the presence of antimycin was 85 nmol of Q/min per mg of protein, equal to the uncoupled rate in these conditions. The maximum rate of oxidation in the presence of 5 mM-malonate and 100 pmol of FCCP/mg of protein was 185 nmol of QH₂/min per mg of protein, and the rate was first-order with respect to Q-2 concentration. These rates are sufficient to suggest that all electron flow may pass through Q-2 rather than endogenous Q-10, at least up to the uncoupled rate. However, we cannot discount the possibility that Q-2 is in rapid equilibrium with Q-10 either directly or through the *o* site of the cytochrome *bc*₁ complex, or via succinate dehydrogenase.

The rotenone by-pass of site 1 had a maximum rate of 1 nmol of O/min per mg of protein, from endogenous substrates in the presence of 10 μ M-Q-2 and 5 μ M-rotenone. There was no measurable by-pass of the antimycin-sensitive site between Q-2 and exogenous cytochrome *c* in these conditions, as assessed by antimycin-inhibited respiration and by

cytochrome *c* reduction in the presence of cyanide and succinate. Thus in the steady state the rate of O_2 consumption was equal to the rate of electron flow through complex 3.

The addition of exogenous horse heart cytochrome *c* ($10\ \mu\text{M}$) increased both the coupled and the uncoupled respiration rates by about 5%. The maximum rate of reduction of exogenous cytochrome *c* monitored spectrophotometrically at 550 nm–540 nm in the presence of excess succinate, FCCP and cyanide was equivalent to 5 nmol of O /min per mg of protein. Experiments (not shown) with isolated mitoplasts (outer membrane removed

with digitonin) gave $\Delta\psi$ greater than 150 mV and graphs of $\Delta E_h - \Delta p$ versus J_O identical with those for whole mitochondria shown below, and so for convenience most experiments were done with whole mitochondria.

We thus concluded that the experimental system used in the presence of exogenous Q-2 and cytochrome *c* was suitable for the purposes of the following experiments.

FCCP titration of $\Delta\psi$, ΔE_h and J_O

To discover how the force ratio $\Delta E_h/\Delta p$ and disequilibrium $\Delta E_h - n \cdot \Delta p$ between Q-2 and cytochrome *c* changed as $\Delta\psi$ was lowered and J_O increased, FCCP was titrated into State 4 mitochondria respiring on succinate in the presence of Q-2 and cytochrome *c*. $\Delta\psi$ was followed either with a TPMP⁺ electrode or by using the isotopic label [³H]TPMP⁺, which gave the same results. $\Delta p\text{H}$ was decreased by including 120 mM-KCl and nigericin, as well as 5 mM-phosphate. Fig. 2 shows the change in $E_h(\text{Q-2})$, $E_h(\text{cyt. } c)$, $\Delta\psi$ and $-59\Delta p\text{H}$, plotted against rate of O_2 consumption (J_O), as the rate was titrated with FCCP. Since all electron flow passes through the bc_1 complex, J_O was equal to electron flow through the cytochrome bc_1 complex in the steady state. In Fig. 3 ΔE_h , Δp , $\Delta E_h/\Delta p$ and $\Delta E_h - \Delta p$ are plotted against J_O . The force ratio $\Delta E_h/\Delta p$ in State 4 was $1.03 (\pm 0.04)$ and extrapolated back to a value close to 1 (0.96) at zero electron flow. At equilibrium we know that, since ΔG of the reaction is zero, then $\Delta E_h - n \cdot \Delta p = 0$, thus $n = \Delta E_h/\Delta p$, and therefore the vectorial H^+/e^- stoichiometry is 1:1. If we now assume this

Fig. 2. Redox states of Q-2 and cytochrome *c* in rat liver mitochondria as $\Delta\psi$ and J_O are titrated with FCCP. Mitochondria (1 mg of mitochondrial protein/ml) were incubated in medium containing 120 mM-KCl, 5 mM-phosphate (potassium salt), 5 mM-Hepes (potassium salt), 5 mM-mannitol, 1 mM-EGTA (potassium salt), 100 μM -potassium acetate, 10 μM -Q-2, 5 μM -rotenone, 5 μM -TPMP⁺ bromide, 3.3 μM -cytochrome *c*, 0.6 μg of oligomycin/ml and 0.3 μg of nigericin/ml, pH 7.00, at 25°C, for 2 min. Mitochondria were then energized with 5 mM-succinate (potassium salt), and $\Delta\psi$ was decreased with successive additions of 16.7 pmol of FCCP/mg of protein, at 2 min intervals when a steady state was reached, up to a final concentration of 66.7 pmol of FCCP/mg of protein. Parallel determinations were made in each steady state of matrix volume, $\Delta\psi$ (●), $-\Delta p\text{H}$ (○), $E_h(\text{Q-2})$ (□), $E_h(\text{cyt. } c)$ (■) and rate of O_2 consumption, as described in the Experimental section. Each point is the mean for three experiments of triplicate determinations. The bars represent $\pm 1\text{s.d.}$ of the means for three experiments. The $E_h(\text{Q-2})$ is calculated with respect to the external pH, assuming an $E_m^0(\text{Q-2, pH 7.0})$ value of +100 mV. The matrix volume averaged at $0.46\ \mu\text{l}/\text{mg}$ of protein.

Fig. 3. ΔE_h , Δp , $\Delta E_h/\Delta p$ and $\Delta E_h - \Delta p$ across the cytochrome bc_1 complex of rat liver mitochondria, plotted against respiration rate (J_O), calculated from the values in Fig. 2.

ΔE_h is $E_h(\text{cyt. } c) - E_h(\text{Q-2})$ (●); Δp is ($\Delta\psi - 59\Delta p\text{H}$) (○); $\Delta E_h/\Delta p$ (■) is the force ratio; $\Delta E_h - \Delta p$ (□) is equal to $(-\Delta G/F)$ for electron flow through the cytochrome bc_1 complex coupled to vectorial proton transport, assuming a constant stoichiometry of 1 H^+/e^- .

stoichiometry over the whole range of rates, then $\Delta G = -F(\Delta E_h - 1 \cdot \Delta p)$. From Fig. 3 we see that $-\Delta G/F$ increases approximately linearly with rate, almost up to the uncoupled rate.

Note that we have calculated $E_m(Q-2)$ as $E_o(Q-2) - 59\text{pH}$, using the external pH. This assumes that ubiquinol released its two protons to the external medium when donating electrons to the cytochrome bc_1 complex. Thus these protons are excluded from the calculation of $[E_h(\text{cyt. } c) - E_h(Q-2)]/\Delta p$, which reflects only the stoichiometry of protons actually pumped by the cytochrome bc_1 complex. At equilibrium the difference between the E_h of the Q-2 couple receiving electrons from succinate dehydrogenase (Sd) and protons from the inside of the membrane

$E_h(\text{Sd})$, and the Q-2 donating electrons to the cytochrome bc_1 complex (cyt. bc_1) and protons to the outside of the membrane $E_h(\text{cyt. } bc_1)$, is equal to $59\Delta\text{pH}$:

$$E_h(\text{cyt. } bc_1) - E_h(\text{Sd}) = -59\Delta\text{pH}$$

Now, out of equilibrium we must have $E_h(\text{cyt. } bc_1) - E_h(\text{Sd}) > -59\Delta\text{pH}$ in order for electrons to go forward from succinate dehydrogenase to the cytochrome bc_1 complex. This disequilibrium will be due to Q diffusion, and will only be significant if Q diffusion is slow relative to overall electron-transport rates. Then, if our spectrophotometric assay of the ratio Q/QH₂ is an average of that at succinate dehydrogenase and that at the cytochrome bc_1 complex, our calculated value of $E_h(Q-2)$ (average, external pH) will be lower than the actual value at the cytochrome bc_1 complex. Thus our value of $\Delta E_h - \Delta p$ will include a component due to any rate limitation in Q diffusion.

FCCP titration of ΔpH , Δp , ΔE_h and J_o

In order to discover how ΔpH affects the rate through the cytochrome bc_1 complex, mitochondria were incubated in a medium that effectively clamps $\Delta\psi$, i.e. 5 mM-KCl plus valinomycin. J_o and Δp were then titrated with FCCP as above. Fig. 4 shows the values of $E_h(Q-2)$, $E_h(\text{cyt. } c)$, $\Delta\psi$ and ΔpH against rate. Fig. 5 shows that $\Delta E_h/\Delta p$ was $1.25 (\pm 0.05)$ in State 4 and extrapolates to 1.1 at zero rate; then, if we assume an H^+/e^- stoichiometry of 1:1 as before, the plot of ΔG against J_o is roughly linear but does not pass through the origin.

Fig. 4. Redox states of Q-2 and cytochrome c of rat liver mitochondria as ΔpH and J_o are titrated with FCCP. Mitochondria (1 mg of mitochondrial protein/ml) were incubated in medium containing 200 mM-sucrose, 5 mM-KCl, 5 mM-Hepes (lithium salt), 5 mM-mannitol, 1 mM-EGTA (lithium salt), 100 μM -potassium acetate, 10 μM -Q-2, 5 μM -rotenone, 5 μM -TPMP⁺ bromide, 3.3 μM -cytochrome c , 0.6 μg of oligomycin/ml and 320 pmol of valinomycin/mg of protein, pH 7.00, at 25°C, for 2 min. Mitochondria were then energized with 5 mM-succinate (lithium salt), and ΔpH was decreased with successive additions of 16.7 pmol of FCCP/mg of protein, at 2 min intervals when a steady state was reached, up to a final concentration of 66.7 pmol of FCCP/mg of protein. Parallel determinations were made in each steady state as before. The points represent the redox state $E_h(Q-2)$ ($\square$), $E_h(\text{cyt. } c)$ ($\blacksquare$), the membrane potential $\Delta\psi$ ($\bullet$) and $-59\Delta\text{pH}$ ($\circ$). Each point is the mean for three experiments of triplicate determinations. The bars represent ± 1 S.D. of the means for three experiments. $E_h(Q-2)$ is calculated with respect to the external pH, assuming an $E_m(Q-2, \text{pH } 7.0)$ value of +100 mV. The matrix volume averaged at 0.78 μl /mg of protein.

Fig. 5. ΔE_h , Δp , $\Delta E_h/\Delta p$ and $\Delta E_h - \Delta p$ across the cytochrome bc_1 complex of rat liver mitochondria, against respiration rate (J_o), calculated from the values in Fig. 4. ΔE_h is $E_h(\text{cyt. } c) - E_h(Q-2)$ ($\bullet$); Δp is $(\Delta\psi - 59\Delta\text{pH})$ ($\circ$); $\Delta E_h/\Delta p$ ($\blacksquare$) is the force ratio; $\Delta E_h - \Delta p$ ($\square$) is equal to $(-\Delta G/F)$ for electron flow through the cytochrome bc_1 complex coupled to vectorial proton transport, assuming a constant stoichiometry of 1 H^+/e^- .

Duroquinone

Much of this work was repeated with duroquinone in the place of Q-2, though the detailed results are not shown here. Duroquinone (50 μM) was used with 2 mg of mitochondrial protein/ml. The redox state of duroquinone was followed at 265 nm–285 nm. Assuming E'_m to be +46 mV (Clark, 1960), $\Delta E_h/\Delta p$ in State 4 was 1.2 (± 0.2). FCCP titrations of $\Delta\psi$ gave similar results to those with Q-2. However, owing to the lower midpoint potential of duroquinone, and thus the low percentage of reduced duroquinone, it was difficult to follow the redox state during a titration with FCCP.

Discussion

There are several possible modes by which Q-2 could interact with the respiratory chain between succinate dehydrogenase and the cytochrome bc_1 complex, but, since the rate constants of Q-2 reduction and oxidation are sufficient to account for the whole of the electron flow passing through Q-2 alone, this is not relevant to any general conclusions that we may draw about the thermodynamics of the cytochrome bc_1 complex.

Fig. 2 shows that the force ratio $\Delta E_h/\Delta p$ across the cytochrome bc_1 complex in State 4 is equal to 1.03 (± 0.04), and that this increases as $\Delta\psi$ is decreased and respiration increased by FCCP. At equilibrium the force ratio is equal to the H^+/e^- stoichiometry. This does not include any protons translocated by the pool Q, as these are excluded from the force ratio by using the external pH to calculate the redox potential of Q-2. Thus our thermodynamic estimate of H^+/e^- stoichiometry at complex 3 agrees well with estimates from kinetic methods (Di Virgilio *et al.*, 1981; Alexandre & Lehninger, 1979), which all now agree that 1 H^+/e^- is translocated by pool Q and 1 H^+/e^- by the cytochrome bc_1 complex itself.

If we assume a constant stoichiometry of 1 H^+/e^- for the cytochrome bc_1 complex, for all rates, we can then calculate the disequilibrium (ΔG) across the complex. Fig. 3 shows that ΔG is measurably large and increases (more negative) roughly linearly with rate. Note, however, that the absolute value of ΔG is relatively sensitive to any consistent errors that we have made in measuring matrix volumes, $\Delta\psi$, ΔpH and the redox potentials of Q-2 and cytochrome c .

A linear relationship between ΔG and rate, with large ΔG (ΔG approx. RT), might seem unexpected in terms of enzyme kinetics, but such relationships can be derived from enzyme kinetics under certain boundary conditions, in particular where the sum of substrate and product concentrations is constant

(Van der Meer *et al.*, 1980). A linear relationship between ΔG and the rate of reverse electron transport from succinate to NAD^+ has previously been demonstrated (Rottenberg & Gutman, 1977). Such relationships enable the theory of irreversible thermodynamics to provide a useful description of respiration. Stucki *et al.* (1983) have pointed out that linear relations can provide a much more efficient mode of coupling far from equilibrium.

In Fig. 5, where J_O is increased by decreasing ΔpH , there is a linear relationship between ΔG and J_O of similar slope to that in Fig. 3, where J_O is increased by decreasing $\Delta\psi$. This indicates that internal pH must have some control strength over an internal reaction in the cytochrome bc_1 complex. The fact that the plot of ΔG versus J_O does not extrapolate through zero in Fig. 5 may indicate that we have underestimated ΔpH , or alternatively that internal pH has some additional kinetic control over the cytochrome bc_1 complex. In terms of the Q-cycle model of the cytochrome bc_1 complex (Mitchell, 1976), the reactions involving internal protons are Q reduction by b cytochromes and/or semiquinone dismutation at centre i . However, internal pH might affect rate indirectly via the pH-dependent midpoint potentials of the b cytochromes, or protonation/deprotonation of other subunit proteins.

With regard to electron transport from succinate to Q we can see from Fig. 2 that Q-2 is not at equilibrium with external succinate, as the E'_m (pH 7.0) of succinate/fumarate is equal to +31 mV, and we are adding succinate alone externally. This disequilibrium increases with rate, and in the uncoupled state Q-2 is completely oxidized. The disequilibrium step may be succinate transport, complex 2 and/or Q diffusion. In the ΔpH titration Fig. 4 shows that the redox state of Q-2 changes much less with rate. This may indicate that the presence of phosphate and the absence of ΔpH in the $\Delta\psi$ titration conditions caused some rate limitation of succinate transport. Whichever step is involved, this component must be taken into account in any model of respiratory control. Note, however, that this may be an artifact of Q-2 addition.

Considering electron transport from cytochrome c to O_2 , Fig. 2 shows that as O_2 consumption increases cytochrome c becomes more oxidized in these conditions, i.e. as the rate increases the substrate (cytochrome c^{2+}) concentration for cytochrome c oxidase decreases. This indicates that $\Delta\psi$ must have a strong control strength over electron flow through cytochrome c oxidase. In the ΔpH titration (Fig. 4), as ΔpH decreases and J_O increases, $\Delta\psi$ and the redox state of cytochrome c change very little, and thus ΔpH (or internal pH) must also have some control strength over cyto-

chrome *c* oxidase, although it appears less (per mV) than $\Delta\psi$.

For the whole respiratory span from succinate to O₂, respiratory control can be seen to be a complex process with control exerted at several points along the chain. The linearity between forces and rates of several of the component reactions suggests that irreversible thermodynamics may be useful in describing control. However, a knowledge of the dependence of electron transport rate on E_h is needed before a quantitative model can be proposed. Such a model may also be useful in assessing whether localized circuits of protons may play a role in respiratory control, as proposed by advocates of localized chemiosmosis (Westerhoff *et al.*, 1984).

We thank Dr. Ian Ragan for the gift of Q-2. G. C. B. is grateful to the Medical Research Council for the award of a research studentship.

References

- Alexandre, A. & Lehninger, A. L. (1979) *J. Biol. Chem.* **254**, 11555–11560
- Chance, B. & Williams, G. R. (1955) *J. Biol. Chem.* **217**, 409–427
- Chappell, J. B. & Hansford, R. G. (1972) in *Subcellular Components: Preparation and Fractionation* (Birnie, G. D., ed.), 2nd edn., pp. 77–91, Butterworths, London
- Clark, W. M. (1960) *Oxidation-Reduction Potentials of Organic Systems*, Williams and Wilkins, Baltimore
- De Jonge, P. C. & Westerhoff, H. V. (1982) *Biochem. J.* **204**, 515–523
- Di Virgilio, F., Pozzan, M. & Azzone, G. F. (1981) *Eur. J. Biochem.* **117**, 225–231
- Gornall, A. G., Bardawill, C. J. & David, M. M. (1949) *J. Biol. Chem.* **177**, 751–766
- Gutman, M. & Silman, N. (1975) *Mol. Cell. Biochem.* **7**, 177–185
- Holian, A. & Wilson, D. F. (1980) *Biochemistry* **19**, 4213–4221
- Johnson, R. N. & Hansford, R. G. (1977) *Biochem. J.* **164**, 305–322
- Kamo, N., Muratsugu, M., Hongoh, R. & Kobatake, Y. (1979) *J. Membr. Biol.* **49**, 105–121
- Mitchell, P. (1966) *Biol. Rev. Cambridge Philos. Soc.* **41**, 445–502
- Mitchell, P. (1976) *J. Theor. Biol.* **62**, 327–367
- Nicholls, D. G. (1974) *Eur. J. Biochem.* **49**, 573–584
- Padan, E. & Rottenberg, H. (1973) *Eur. J. Biochem.* **40**, 431–437
- Prince, R. C., Linkletter, S. J. G. & Dutton, P. L. (1981) *Biochim. Biophys. Acta* **635**, 132–148
- Rottenberg, H. (1979) *Biochim. Biophys. Acta* **549**, 225–253
- Rottenberg, H. & Gutman, M. (1977) *Biochemistry* **16**, 3220–3227
- Stucki, J. W., Compiani, M. & Caplan, S. R. (1983) *Biophys. Chem.* **18**, 101–109
- Van der Meer, R., Westerhoff, H. V. & Van Dam, K. (1980) *Biochim. Biophys. Acta* **591**, 488–493
- Westerhoff, H. V., Melandri, B. A., Venturoli, G., Azzone, G. F. & Kell, D. B. (1984) *FEBS Lett.* **165**, 1–5