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Proton/electron stoichiometry of mitochondrial complex I estimated from the equilibrium thermodynamic force ratio

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The thermodynamic forces on electrons (ΔE_h) and protons (Δp) across mitochondrial complexes I, III and IV were measured in isolated mitochondria respiring on succinate. The force ratio ($\Delta E_h/\Delta p$) across complex I close to equilibrium was found to be about 2. The equilibrium force ratio across complex I was measured during sulphite oxidation and was again close to 2. These results indicate that the proton/electron stoichiometry of complex I is 2, in conditions of high protonmotive force.

INTRODUCTION

The proton/electron (H^+/e) stoichiometry (protons released externally per electron) and q^+/e stoichiometry (net positive charge transported out per electron) of NADH:ubiquinone oxidoreductase (complex I) of the mitochondrial respiratory chain are still controversial. Indirect kinetic estimates, calculated by subtracting the H^+/e estimate for mitochondria respiring on succinate from the estimate for mitochondria respiring on NAD^+-linked substrates have given H^+/e values from 1 to 2 (H^+/e values: 1.1, Al-Shawi & Brand, 1981; 1.4, Brand *et al.*, 1976; 1.8, Pozzan *et al.*, 1979; 2.0, Vercesi *et al.*, 1978; 2.0, Beavis, 1987*a*). The most direct and extensive kinetic investigation, using ubiquinone-1 pulses, gave estimates of about 1.8 H^+/e and q^+/e for complex I (Di Virgilio & Azzone, 1982), leading the authors to conclude that the mechanistic stoichiometry was 2 H^+/e. However De Jonge & Westerhoff (1982) concluded that the true stoichiometry was 1.5 H^+/e, from estimates of the $\Delta E_h/\Delta p$ thermodynamic force ratio across complex I close to equilibrium in submitochondrial particles. On the other hand these authors did not take into account binding of the probes for Δp in submitochondrial particles which has since been shown to be significant (Berry & Hinkle, 1983). We therefore undertook to measure the force ratio across complex I close to equilibrium in mitochondria, in order to obtain a reliable estimate of the H^+/e stoichiometry in conditions of high Δp. We report here the force ratios across complexes I, III and IV for intact mitochondria in static head respiring on succinate, and the equilibrium force ratio across complex I for mitochondria respiring on sulphite.

EXPERIMENTAL

Liver mitochondria were prepared from 200–250 g female Wistar rats by standard procedures (Chappell & Hansford, 1972) in 250 mM-sucrose/5 mM-Tris/HCl/1 mM-EGTA at pH 7.4. The protein concentration of the preparation was estimated by a Biuret method (Gornall *et al.*, 1949) against a bovine serum albumin standard.

Mitochondria were then incubated in the media and at the temperature indicated in the Figure legends, respiring either on succinate or sulphite, and at the stated time the components of the protonmotive force (Δp) and redox potentials (E_h) were determined.

The components of Δp were determined by including the appropriate isotopes in the incubation medium, and centrifuging the incubation to assay the distribution of isotopes between supernatant and pellet, as in Murphy & Brand (1987). Matrix volume was estimated from the difference between uptake into the pellet of either [^{14}C]mannitol (0.2 μCi/ml) or [^{14}C]sucrose (0.2 μCi/ml) as against ^3H_2O (5.0 μCi/ml). ΔpH was estimated using [^{3}H]acetate (5.0 μCi/ml) and [^{14}C]mannitol (0.2 μCi/ml), and calculated from the accumulation of acetate into the matrix volume (estimated with mannitol). $\Delta\psi$ was estimated using [^{3}H]TPMP$^+$ (0.1 μCi/ml). Methyltriphenylphosphonium ion (TPMP$^+$) accumulation into the mitochondria was calculated from the difference between the uptake of TPMP$^+$ and mannitol into the pellet, divided by the matrix volume estimated with mannitol. $\Delta\psi$ was calculated as $z\cdot\log$(TPMP$^+$ accumulation $\cdot$ binding constant), where $z = 2.303\boldsymbol{R}\cdot T/F$ and the binding constant is the ratio of $^{86}Rb^+$ to [^{3}H]TPMP$^+$ accumulation at that particular matrix volume, determined in a calibration described in the Results section. TPMP$^+$ accumulation was alternatively determined with a TPMP$^+$-sensitive electrode, as in Brown & Brand (1985). TPMP$^+$ and acetate uptake were determined in triplicate, sucrose and mannitol volumes in sextuplicate.

The redox potential (E_h) on the reducing side of complex I was determined from the equilibrium ratio of acetoacetate (AcAc) to D-3-hydroxybutyrate (D-3-HB), and calculated as

$$E_{M,7} + (z/2)\cdot\log([\text{AcAc}]/[\text{D-3-HB}]).$$

The $E_{M,7}$ was taken as -266 mV at 25 °C and interpolated to be -276 mV at 30 °C and -291 mV at 37 °C

Abbreviations used: AcAc, acetoacetate; D-3-HB, D-3-hydroxybutyrate; e, electron; E_h, actual redox potential; $E_{M,7}$, midpoint potential at pH 7; E_0, redox potential in standard state; ΔE_h, difference in redox potential between two couples; ΔG_p, Gibbs free-energy change of ATP hydrolysis; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; $\Delta\psi$, electrical potential across mitochondrial inner membrane; ΔpH, pH difference across mitochondrial inner membrane; Δp, protonmotive force across mitochondrial inner membrane; Q-2, ubiquinone-2; TPMP$^+$, methyltriphenylphosphonium ion; z, 2.303 $\boldsymbol{R}\cdot T/F$.

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(Hoff-Jorgensen, 1938). The mitochondrial incubation was centrifuged at 12000 *g* for 2.5 min and the AcAc and D-3-HB concentrations in the supernatant were determined enzymically as in Williamson & Mellanby (1974). The redox potential between complexes I and III (i.e. of the ubiquinone pool) was determined from the steady-state reduction of added ubiquinone-2 (Q-2) and calculated as $E_{M,7}+(z/2)\cdot\log(\%\text{ oxidized Q-2}/\%\text{ reduced Q-2})$, where the $E_{M,7}$ was taken as +112 mV at 25 °C [this is the median of the literature values: 104 mV, Morton (1965); 112 mV, Schnorf (1966) and quoted in Urban & Klingenberg (1969); 126 mV, Moret *et al.*, (1961)] and estimated to be +102 mV at 30 °C and +87 mV at 37 °C, from analogy with benzoquinone systems where $\Delta E_0/\Delta T = -0.74$ mV/degree (Clark, 1960). The reduction state of Q-2 was determined spectrophotometrically at 275–290 nm as in Brown & Brand (1985), but using a 2 mm path length cuvette when using 3 mg of mitochondrial protein/ml. The redox potential between complexes III and IV (i.e. of the cytochrome *c* pool) was determined from the steady-state reduction of endogenous cytochrome c/c_1 measured at 550–540 nm and calculated as $E_{M,7}+z\cdot\log(\%\text{ oxidized}/\%\text{ reduced})$, where the $E_{M,7}$ at 25 °C was taken at +235 mV (Dutton *et al.*, 1970) and estimated to be +229 mV at 30 °C and +221 mV at 37 °C using the value $\Delta S = -114$ J/mol·degree quoted by Langerman (1976) and the relation $\Delta E_0/\Delta T = \Delta S_0/F$. The redox potential on the oxidizing side of complex IV was calculated from the oxygen concentration ($[O_2]$, expressed in μmol/ml) as $E_{0,7}+(z/4)\cdot\log([O_2]/1.2)$ where $E_{0,7}$ was taken as +815 mV at 25 °C, and estimated to be +804 mV at 30 °C and +789 mV at 37 °C using the value $\Delta S_0 = -82$ J/equivalent·degree (George, 1965). All incubations were shaken vigorously to maintain oxygen saturation of the medium between 90 and 20%, as determined by Clark oxygen electrode, averaging around 40% saturation at the end of the incubation, thus the E_h of the oxygen/water couple was calculated for 40% oxygen saturation. Respiration rates were also determined by oxygen electrode.

All redox potentials were calculated using the extramitochondrial pH (pH 7.0), as comparison of ΔE_h and Δp requires the values of E_h to be calculated for the same compartment (see Lemasters *et al.*, 1984). However, due to the fact that both the D-3-HB/AcAc and QH_2-2/Q-2 couples release $2H^+/2e$, at equilibrium, the ΔE_h between the couples is the same inside and outside the mitochondria, irrespective of the ΔpH.

All radioisotopes were from Amersham International (Amersham, Bucks., U.K.) except [^{3}H]TPMP$^+$-bromide which was from New England Nuclear (Dreieich, Germany). Q-2 was a gift of Eisai Co., Tokyo, Japan. Unlabelled TPMP$^+$-bromide and D-3-HB were from Aldrich Chemical Co. (Gillingham, Dorset, U.K.). All other chemicals were from Sigma Chemical Co. (Poole, Dorset, U.K.).

RESULTS

Measurement of mitochondrial volume

As a preliminary to measuring the force ratios, some reassessment of the measurement of Δp in isolated mitochondria was made. Halestrap & Quinlan (1983) have asserted that sucrose is unsuitable as an extramitochondrial marker for matrix volume determination,

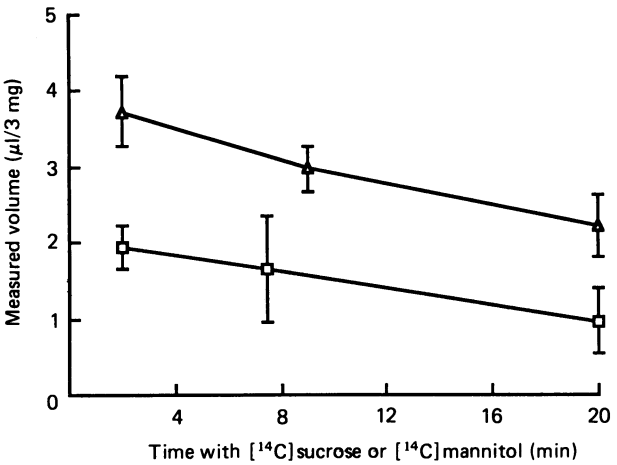

Fig. 1. Measured [^{14}C]sucrose and [^{14}C]mannitol excluding volumes as a function of time in the presence of the isotopes

Mitochondria (3 mg of protein/ml) were incubated in 200 mM-sucrose/20 mM-KCl/5 mM-Hepes/5 mM-succinate (potassium salt)/1 mM-malonate (potassium salt)/1 mM-EGTA, adjusted to pH 7.0 with KOH, plus 100 μM-sodium acetate, 20 μM-Q-2, 5 μM-TPMP-bromide and ^3H_2O. Incubations were at 30 °C for a total of 20 min. Either [^{14}C]sucrose (△) or [^{14}C]mannitol (□) was added for the indicated times before terminating the incubation by centrifugation. Error bars show one S.D. on either side of the mean of six replicates on the same mitochondrial preparation.

on the basis that mannitol rapidly penetrates into part of the volume which excludes sucrose, and the mannitol-excluding volume extrapolates to zero at infinite osmolarity, whereas the sucrose-excluding volume does not. We therefore determined both volumes for mitochondria incubated in the conditions used for the assay of the force ratio, as a function of the time in the presence of the isotopes. That is, the mitochondria were incubated at 30 °C in the indicated media for 20 min with ^3H_2O, but [^{14}C]mannitol or [^{14}C]sucrose were added to the incubation either 20 min, 8 min or 2 min before termination of the incubation by centrifugation. Fig. 1 shows that both mannitol and sucrose slowly penetrate into mitochondria in these conditions, but that mannitol rapidly penetrates a volume that excludes sucrose. As the measured mannitol-excluding volume was unaffected by raising the unlabelled mannitol concentration from 4 μM to 100 mM (replacing sucrose for the same osmolarity), it was concluded that the difference in measured volume was not due to mannitol binding. The mannitol-excluding volume was also found to extrapolate to roughly zero at infinite osmolarity of the incubation medium, thus in subsequent determinations of matrix volume mannitol was used (added 2 min before centrifugation).

Mannitol rapidly penetrates a compartment which excludes sucrose. The nature of this compartment is unknown; it might be an identifiable ultrastructural feature of all mitochondria, such as the cristae, or a subpopulation of mitochondria, or contaminating vesicles. Both mannitol and sucrose appear to slowly penetrate the total mitochondrial volume. This has been reported previously for mannitol (Halestrap & Quinlan, 1983) but not for sucrose, although sucrose penetration has been reported to be affected by phosphate (Rotten-

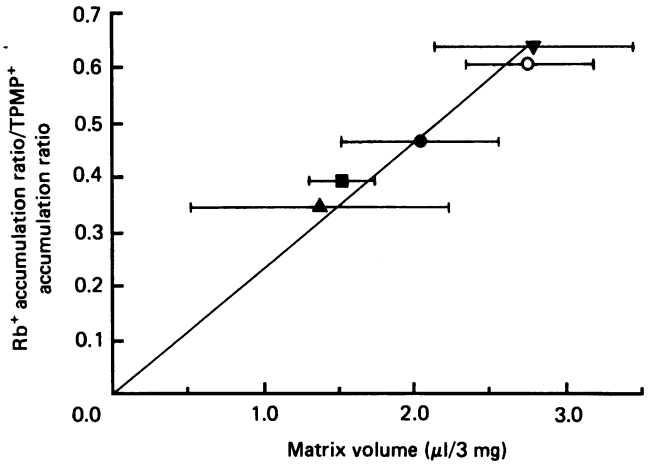

Fig. 2. Ratio of Rb^+ to $TPMP^+$ accumulation as a function of matrix volume

Mitochondria (3 mg of protein/ml) were incubated for 2.5 min at 30 °C in 5 mM-Hepes/5 mM-succinate (lithium salt)/1 mM-mannitol/1 mM-EGTA, adjusted to pH 7.0 with LiOH, plus 5 μM-TPMP-bromide, 2.5 μM-rotenone, 0.6 μg of valinomycin/ml, 0.6 μCi of [^{3}H]$TPMP^+$/ml, 0.04 μCi of $^{86}Rb^+$/ml, plus either 100 mM (▼), 180 mM (●), 240 mM (■) or 300 mM (▲) sucrose with no added KCl, or 180 mM-sucrose and 0.6 mM-KCl (○). Matrix volume was determined in parallel, with ^3H_2O and [^{14}C]mannitol replacing [^{3}H]$TPMP^+$ and $^{86}Rb^+$. Error bars show one S.D. on either side of the mean of six replicate determinations of volume on the same preparation of mitochondria. Error bars for triplicate determinations of the ratio of accumulations are smaller than the symbols used here. The line was fitted by eye to pass through the origin. An identical experiment on another preparation of mitochondria gave the same slope for the line.

berg & Solomon, 1969), valinomycin (Pietrobon *et al.*, 1982), calcium and lipid peroxidation (Crompton *et al.*, 1987).

Measurement of membrane potential

$TPMP^+$ binds to mitochondria and a variety of corrections for this binding have been suggested, according to various models of the binding (Pietrobon *et al.*, 1982; Rottenberg, 1984; Jackson & Nicholls, 1986). One such correction is essentially to calibrate $TPMP^+$ accumulation against $^{86}Rb^+$ accumulation (in the presence of valinomycin) over a range of $\Delta\psi$ values and assume there is a fixed ratio between $^{86}Rb^+$ accumulation and $TPMP^+$ accumulation in set conditions (for example: Scott & Nicholls, 1980; Wilson & Forman, 1982; Brown & Brand, 1985). However we have found that this ratio depends on the matrix volume (as one might expect from reasonable models of binding). Mitochondria were incubated in media of varying osmolarity (100–300 mM-sucrose, same ionic strength) with [^{3}H]$TPMP^+$, $^{86}Rb^+$ and valinomycin, and energized with succinate. Fig. 2 shows the ratio of Rb^+ accumulation to $TPMP^+$ accumulation plotted against the mannitol-excluding volume. In the absence of added potassium, the volume was proportional to the inverse of the osmolarity, but Rb^+ accumulation and thus $\Delta\psi$ was roughly constant (varying from 168–173 mV), while the ratio of Rb^+ accumulation to $TPMP^+$ accumulation was roughly proportional to matrix volume. With a small amount of added potassium (0.6 mM) the measured volume increased and $\Delta\psi$ declined (to 145 mV) but the relationship between the ratio of $TPMP^+$ and Rb^+ accumulation and the matrix volume was retained, indicating this relationship is independent of $\Delta\psi$ over the range investigated. Thus this empirical relationship was used to relate measured $TPMP^+$ accumulation (in the absence of valinomycin) to Rb^+ accumulation and thus to $\Delta\psi$ (calculated as in the Experimental section). $TPMP^+$ uptake was also measured with a $TPMP^+$ electrode and this gave almost identical distributions (less than 1 mV difference) to the isotope method. This is an excellent test of the isotope/centrifugation method.

It is not unexpected that the ratio of Rb^+ accumulation to $TPMP^+$ accumulation into mitochondria should depend strongly on the matrix volume, as the free $TPMP^+$ accumulation should be equal to the Rb^+ accumulation, and the ratio of free to bound $TPMP^+$ in the matrix should increase as the matrix volume increases. However the finding that the ratio of Rb^+ to $TPMP^+$ accumulation was roughly proportional to matrix volume is rather unexpected as this has the consequence that $TPMP^+$ uptake is actually independent of the volume over the range studied. This relationship cannot be straightforwardly explained by Rottenberg's model (Rottenberg, 1984) of $TPMP^+$ binding to mitochondria, which assumes a constant partition coefficient of $TPMP^+$ between the matrix and membrane.

Measurement of redox states

In order to measure the force ratio across complex I, in conditions where the complex was close to equilibrium, mitochondria were allowed to respire on succinate, but with sufficient malonate present to partially inhibit succinate dehydrogenase and roughly half oxidize the Q-2, so that its redox state, and that of the AcAc/D-3-HB couple, could be measured accurately. The redox state on the reducing side of complex I was estimated by adding 50 μM-AcAc and 50 μM-D-3-HB and measuring the equilibrium ratio. These ketone bodies are thought to cross the inner mitochondrial membrane electroneutrally as the protonated acid (Moyle & Mitchell, 1973) and equilibrate with matrix NAD^+/NADH via D-3-HB dehydrogenase located on the matrix side of the membrane (Williamson *et al.*, 1967). Fig. 3 shows the time course of the ketone body equilibration, starting with either 100 μM-AcAc alone or 100 μM-D-3-HB alone; the same equilibrium ratio of AcAc/D-3-HB was approached from either starting point. This ratio was increased by increasing the malonate concentration or including FCCP in the medium, and decreased by reducing the malonate concentration (not shown). The rate of equilibration was inhibited by including rotenone in the medium, and the equilibrium ratio approached was low. There was a small net increase in ketone bodies during the incubation, due to endogenous production.

Q-2 is a short-chain analogue of the endogenous ubiquinone-10, and can interact directly and rapidly with complexes I, II and III at their quinone binding sites (Crane, 1977; Cabrini *et al.*, 1981) and thus may be used to estimate the redox potential of the quinone pool between these complexes (Brown & Brand, 1985). Fig. 4 shows a trace of the Q-2 signal at 275–290 nm. The signal corresponding to fully oxidized Q-2 is found by adding excess uncoupler (which gives the same signal changes as

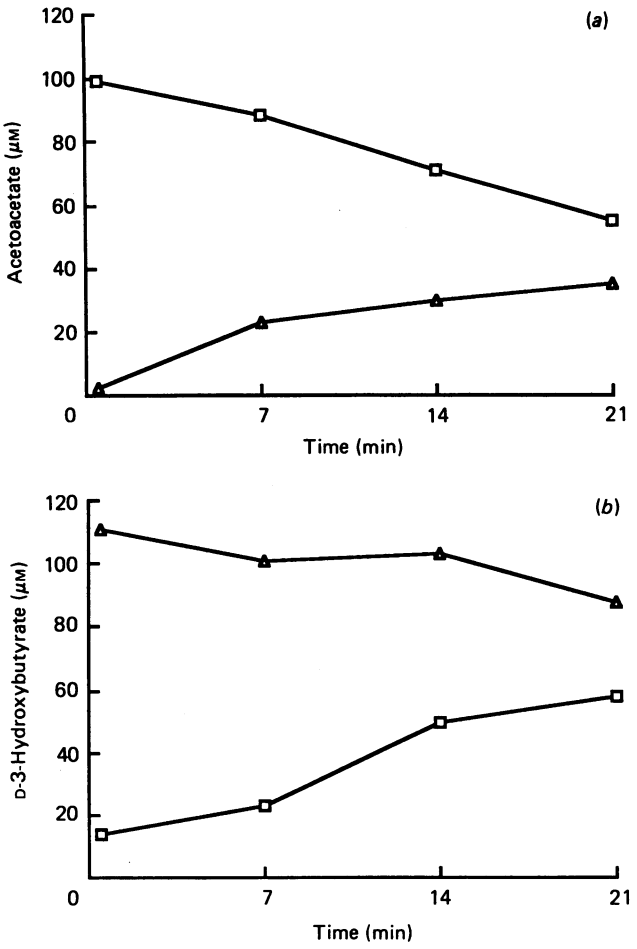

Fig. 3. AcAc concentrations (*a*) and D-3-HB concentrations (*b*) in the medium as a function of time

Mitochondria (3 mg of protein/ml) were incubated for 21 min at 30 °C in 200 mM-sucrose/20 mM-KCl/5 mM-Hepes/5 mM-succinate (potassium salt)/1 mM-malonate (potassium salt)/1 mM-EGTA, adjusted to pH 7.0 with KOH, plus 100 μM-sodium acetate, 20 μM-Q-2, 5 μM-TPMP-bromide, plus either 100 μM-AcAc (lithium salt, □) or 100 μM-D-3-HB (sodium salt, △). At the times indicated samples were removed, centrifuged, and the AcAc and D-3-HB concentrations in the supernatant were assayed. These time courses were repeated on every mitochondrial preparation used for assaying the force ratio with succinate.

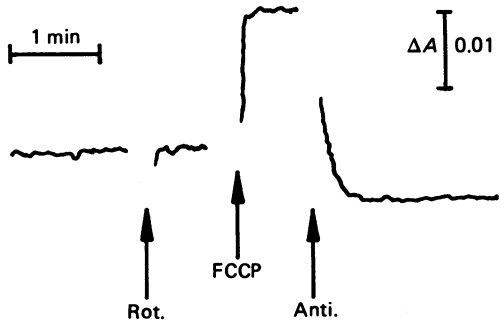

Fig. 4. Trace of 275–290 nm absorbance against time, showing the reduction state of Q-2 during succinate oxidation

Mitochondria were incubated as described in Fig. 3 but with 50 μM-AcAc and 50 μM-D-3-HB, and after 20 min the following additions were made (where indicated by arrows): 0.5 μM-rotenone (Rot.), followed by 0.2 μM-FCCP to fully oxidize, and 1.2 μM-antimycin (Anti.) to fully reduce the Q-2. A representative trace is shown. Absorbance changes in the absence of either Q-2 or mitochondria were negligible. Three such determinations were made per mitochondrial preparation.

excess rotenone and malonate) and the signal corresponding to fully reduced Q-2 was found by adding excess antimycin. As well as interacting at the normal quinone binding sites, Q-2 appears to interact elsewhere in complex I, since the addition of Q-2 to mitochondria induces a rotenone-insensitive electron flux through complex I which is probably not coupled to proton transport, by analogy with the rotenone-insensitive electron flux induced by ubiquinone-0 and ubiquinone-1 (Di Virgilio & Azzone, 1982). The rate of this pathway was measured with an oxygen electrode, in the conditions used for the assay of the force ratio, except with 10 mM-D-3-HB replacing succinate as substrate and in the presence of excess uncoupler (100 pmol of FCCP/mg of protein). The uncoupled rate of oxygen consumption was inhibited 95% by rotenone, thus the rotenone-insensitive pathway contributes 5% or less to the maximum rate. It may be less than this, as the uncoupled rate in the absence of rotenone may not be rate limited by complex I and the sensitive pathway may compete for electrons with the insensitive pathway. Because the rotenone-sensitive pathway has a much greater maximal rate it is to be expected that in the steady state, close to equilibrium, the force ratio across complex I will be much closer to the equilibrium force ratio of the rotenone-sensitive pathway than that of the insensitive pathway. In order to establish the net rate through the rotenone-sensitive pathway in the actual conditions used to assay the force ratio, rotenone was added at the time at which the various parameters were normally assayed. Rotenone addition then resulted in a small or no change in respiration (ranging from 7% stimulation to 5% inhibition of the oxygen consumption rate, which was about 20 nmol of O/min/mg of protein). It also caused from 0 to 8% oxidation at Q-2 (that is % of the total signal, Fig. 4), and no detectable change in the AcAc/D-3-HB ratio. This suggests that, in the steady state before rotenone addition, the net flux through the rotenone-sensitive pathway is small (of the order of 2 nmol of e/min/mg of protein), so that the complex was close to or at equilibrium.

Force ratios under different conditions

Table 1 gives the redox potentials, components of Δp and force ratio assayed after a 20 min incubation at 30 °C with succinate. The force ratio was found to be 2.13 ± 0.12.

The force ratio was also assayed at 37 °C as the equilibration of ketone bodies is much faster at this temperature; the AcAc/D-3-HB ratio was the same after about 10–15 min whether starting from 100 μM-AcAc or 100 μM-D-3-HB. The force ratio was again close to 2 (Table 2).

The redox potential between complexes III and IV was also assayed at this temperature using the endogenous cytochrome c/c_1 signal in order to estimate the force ratios across complex III (the cytochrome bc_1 complex)

Table 1. Redox potentials, Δp and force ratio across complex I at 30 °C

Mitochondria were incubated in the medium given in the legend to Fig. 3. All parameters were determined in parallel after 20 min incubations. The figures are means (±S.E.M.) of three experiments with different mitochondrial preparations.

E_h(AcAc/D-3-HB) = −287 mV (±5)
E_h(Q-2/QH_2-2) = +87 mV (±6)
Matrix volume = 0.65 μl/mg (±0.01)
ΔpH = 20 mV (±3)
$\Delta\psi$ = 156 mV (±6) Δp = 176 mV (±7)
ΔE_h = 374 mV (±10) $\Delta E_h/\Delta p$ = 2.13 (±0.12)

Table 2. Force ratios across complexes I, III and IV at 37 °C

Mitochondria were incubated in the medium given in the legend to Fig. 3, but at 37 °C, and with sufficient malonate to half oxidize the Q-2 (from 0.5 to 1.0 mM-malonate). Redox states and components of Δp were determined in parallel after 15 min incubation. The figures are means (±S.E.M.) of four experiments with different mitochondrial preparations. The cytochrome c/c_1 was 5.3% (±0.3) reduced and Δp ranged from 170–190 mV.

	$\Delta E_h/\Delta p$
Complex I	2.17±0.11
Complex III	1.16±0.04
Complex IV	2.64±0.13

and complex IV (cytochrome oxidase) for comparison. Table 2 shows these force ratios at 37 °C. As there was net forward electron flow through these complexes, the force ratios across complexes III and IV give upper thermodynamic limits to the H^+/e stoichiometry. The actual value of the stoichiometry can only be estimated at equilibrium. This is readily appreciated if the free energy drop (ΔG) across a proton pump is expressed in terms of the thermodynamic forces on the electrons and protons (ΔE_h and Δp) and the proton to electron stoichiometry (n), where:

$$\Delta G = -F(\Delta E_h - n \cdot \Delta p)$$

Experiments were also performed using sulphite instead of succinate to generate Δp. Sulphite is oxidized by sulphite oxidase (located in the intermembrane space) which is then able to reduce cytochrome c, which in turn is oxidized by cytochrome oxidase, generating a Δp (Cohen *et al.*, 1972). Sulphite has a theoretical advantage over succinate oxidation, in that in the presence of myxothiazol to inhibit complex III, it cannot reduce matrix NAD^+ other than via reversal of complex I.

Mitochondria were incubated with Q-2 and also with three different ratios of D-3-HB to AcAc at relatively high concentrations in order to fix the redox potential on the reducing side of complex I. After 2 min, when Q-2 was fully oxidized, excess myxothiazol was added, and Q-2 was reduced at a rate dependent on the ratio of D-3-HB/AcAc (higher rates for a higher ratio). 5 min later (by which time Q-2 was fully reduced) sulphite was added, which induced a Δp and caused partial oxidation of the Q-2. After a further 5 min the equilibrium redox state of Q-2 was taken and in parallel the Δp was measured. Addition of rotenone at this time caused no immediate change in Q-2 reduction (although Q-2 was very slowly reduced over about 10 min), indicating that the complex was essentially at equilibrium. Addition of myxothiazol after sulphite (rather than before) resulted in the same equilibrium redox state of Q-2, although equilibrium was approached from the fully oxidized state of Q-2, rather than the fully reduced state, showing that the same equilibrium could be reached from either side. If excess FCCP or rotenone was present in the medium there was no oxidation on sulphite addition, indicating that the oxidation was due to the Δp inducing reversal of electron flux through complex I. Addition of sulphite to Q-2 in the absence of mitochondria caused no change in the Q-2 spectrum, indicating that there was no direct reduction by sulphite.

Table 3. Thermodynamic forces across complex I during sulphite oxidation

Mitochondria (1 mg of protein/ml) were incubated at 25 °C in 100 mM-KCl/5 mM-Hepes/5 mM-sucrose/1 mM-EGTA/10 μM-potassium acetate/5 μM-TPMP-bromide/10 μM-Q-2/0.5 μM-myxothiazol, adjusted to pH 7.0 with KOH, plus either (*a*) 8 mM-DL-3-HB (potassium salt, adjusted to pH 7.0 with KOH, racemic) and 2 mM-AcAc (lithium salt, adjusted to pH 7.0 with LiOH), or (*b*) 5 mM-DL-3-HB and 5 mM-AcAc or (*c*) 2 mM-DL-3-HB and 8 mM-AcAc. After 5 min preincubation 3 mM-sodium sulphite (adjusted to pH 7.0 with NaOH) was added, and after a further 5 min, the redox state of Q-2 was assayed, and Δp was determined with isotopes. E_h(AcAc/HB) refers to the E_h of the AcAc/D-3-HB couple, and was calculated from the added concentrations. Figures are means (±one S.D.) of three replicates. Units are mV except for the force ratio across complex I ($\Delta E_h/\Delta p$) which is dimensionless.

Condition	E_h(AcAc/HB)(mV)	E_h(Q/QH_2) (mV)	ΔE_h	Δp	$\Delta E_h/\Delta p$
(*a*)	−275	+91 (±1)	366	187 (±4)	1.96
(*b*)	−257	+101 (±1)	359	188 (±4)	1.91
(*c*)	−239	+113 (±2)	353	187 (±6)	1.89

Table 3 shows the measured equilibrium ΔE_h (D-3-HB/AcAc to Q-2), Δp and force ratio in the presence of different ratios of added D-3-HB/AcAc. As expected the redox state of Q-2 changes with the redox potential of the applied D-3-HB/AcAc couple. However it does not change to the same extent, thus the force ratio changes slightly with the D-3-HB/AcAc ratio. This might have been caused by the ketone bodies not being kinetically competent to fully buffer changes in the matrix NAD^+/NADH.

DISCUSSION

The force ratio across complex I was estimated here to be 2.17 (±0.11) when mitochondria were respiring on succinate at 37 °C, 2.13 (±0.12) when they were respiring on succinate at 30 °C, and 1.92 (±0.04) when respiring on sulphite at 25 °C. Since in these conditions the

complex was close to or at equilibrium, these measurements support a stoichiometry of close to $2H^+/e$ for complex I.

The evidence that the complex was close to or at equilibrium is essentially that inhibiting the complex with rotenone caused minimal change in respiration on succinate (about 1 nmol O/min per mg of protein) and minimal change in the redox states of Q-2 and AcAc/D-3-HB. These tests are not quantitative as both the Q-2 redox state and the respiration rate are likely to be buffered by succinate dehydrogenase activity when respiring on succinate, but they do suggest that any flux is small. In the case of sulphite oxidation in the presence of myxothiazol, the fact that rotenone addition causes no immediate change in Q-2 redox state is good evidence that complex I is at equilibrium.

It is difficult to estimate the errors in the force ratio measurements, but any errors in the estimation of the ratio of AcAc/D-3-HB and Q-2/QH_2-2 are likely to be insignificant in terms of the force ratio. The midpoint potential of the AcAc/D-3-HB couple seems to be settled (Krebs *et al.*, 1962). The midpoint potential of the ubiquinone couple used here is the median of literature values (see Experimental section); if instead we used the most extreme literature value of +126 mV the estimated force ratio would change by only about 4%. Errors in estimation of matrix volume, acetate and $TPMP^+$ uptake, and $TPMP^+$ binding have relatively little effect on the force ratio as they appear as logarithmic terms in the Δp estimate. Decreasing any one of these parameters by 50% or increasing it by 100% changes the force ratio estimate by about 10%, which would not affect the likely H^+/e stoichiometry.

Unlike kinetic estimates, equilibrium thermodynamic estimates of stoichiometry are unaffected by proton and other charge permeabilities, and are minimally affected by any heterogeneity of the mitochondrial population.

As a cross-check of the methods, we can get a rough estimate of the H^+/e stoichiometry of complex I, independent of the estimate of Δp, by comparing the force ratio or ΔE_h across complexes I and III. If we assume that the force ratio across complex III in the static head conditions used is close to the equilibrium value as seems likely from Brown & Brand (1985) and Brand *et al.* (1986) and that the stoichiometry of complex III is $1q^+/e$, then the ratio of force ratios (or ΔE_h values) of complex I and III should be just below the H^+/e stoichiometry of complex I. From Table 2 this ratio is 1.87. This is again consistent with a stoichiometry of $2\ H^+/e$ for complex I.

De Jonge & Westerhoff (1982) estimated the $\Delta E_h/\Delta p$ force ratio across complex I during reverse electron transfer in submitochondrial particles and observed values of about 1.6 in static head conditions. They concluded that the H^+/e stoichiometry was 1.5, however they did not take into account binding of the probes for Δp in submitochondrial particles which have since been shown to be significant (Berry & Hinkle, 1983). Therefore De Jonge & Westerhoff (1982) probably overestimated Δp and thus underestimated the force ratio. Di Virgilio & Azzone (1982) made a very rough estimate of the force ratio across complex I in static head for D-3-HB oxidation in mitochondria, and found the value consistent with a stoichiometry of $2H^+/e$, however they did not actually measure the redox potentials or matrix volume, or correct for $TPMP^+$ binding, nor did they show that the complex was near to equilibrium. Freeman & Lemasters (1984) found the $2\Delta E_h/\Delta G_p$ force ratio (i.e. per 2 electrons) across site 1 to be up to 1.18 during reverse electron transfer in mitochondria, thus giving a lower limit to the ATP/2e stoichiometry of 1.18. They concluded that assuming the net H^+/ATP stoichiometry was 4, the likely H^+/e stoichiometry of complex I was 2.5. However Rottenberg & Gutman (1977) and Scholes & Hinkle (1984) both measured the static head $2\Delta E_h/\Delta G_p$ force ratio across site 1 in submitochondrial particles to be close to 4/3, and as the evidence that the H^+/ATP stoichiometry is 3 in submitochondrial particles is fairly good, this suggests an H^+/e stoichiometry of 2 for complex I.

A stoichiometry of 2 is consistent with the most direct and extensive kinetic estimates (Di Virgilio & Azzone, 1982), and with the comparative method of Wikstrom (1984).

The force ratio across complex III (the cytochrome bc_1 complex) was estimated here to be around 1.16 in static head conditions, which is similar to previous estimates made by us (Brown & Brand, 1985; Brand *et al.*, 1986). This then gives an upper limit to the H^+/e stoichiometry of complex III and is consistent with the accepted stoichiometry for the cytochrome bc_1 complex of $1q^+/e$ (releasing $2\ H^+/e$ externally).

The force ratio across complex IV (cytochrome oxidase) was estimated here to be 2.64 in static head conditions, and this again gives an upper limit to the H^+/e stoichiometry and excludes the possibility that cytochrome oxidase transports $3\ q^+/e$ (releasing $2\ H^+/e$ externally), at least at high Δp, as reported by us previously (Murphy *et al.*, 1985). This is consistent with kinetic estimates of the stoichiometry as $2\ q^+/e$ ($1\ H^+/e$ released externally) (Wikstrom, 1977), or $2.5\ q^+/e$ ($1.5\ H^+/e$ released externally), (Beavis, 1987*b*), measured at low Δp.

These observations are not inconsistent with the recent confirmation from this laboratory that q^+/e ratios for succinate oxidation are greatly reduced at high Δp (Murphy & Brand, 1987). The present results show no evidence for slip in complex I or complex III at high Δp, but do not rule out the extensive slip in the non-equilibrium reaction catalysed by complex IV, shown by other methods (Murphy, M. P. & Brand, M. D., unpublished observations).

Overall this work points strongly towards q^+/e ratios of 2 for complex I and 1 for complex III, and is consistent with either a fixed or variable q^+/e stoichiometry in complex IV, with q^+/e values between 1 and 2.5.

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