Proton Translocation Coupled to Quinol Oxidation in Ox Heart Mitochondria

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The suitability of ubiquinol₁ and duroquinol as pulse reductants for initiating respiration-driven proton translocation by aerobic ox heart mitochondria was investigated. At 25°C the $V_{\max}$ for oxidation was close to 280 nmol of quinol oxidized/min per mg of protein, and the K_m values were 8 μ M for ubiquinol₁ and 28 μ M for duroquinol. Pulses of ubiquinol₁ and duroquinol were rapidly and completely oxidized by aerobic mitochondria with a simultaneous acidification of the suspending medium as detected with a glass electrode. The $\rightarrow\text{H}^+/\text{2e}^-$ ratios (Mitchell, 1966) calculated from the observed extent of acidification and the amount of quinol added were 3.62 for ubiquinol₁ and 2.98 for duroquinol. These values are underestimates of the true value owing to proton back-flow across the membrane. An analogue computer model was used to correct the observed extent of respiration-driven acidification for proton back-flow. The corrected $\rightarrow\text{H}^+/\text{2e}^-$ values were 4.01 for ubiquinol and 3.86 for duroquinol oxidation. Attempts to measure the rate of proton translocation with a pH-measuring system with a response time of 0.4 s were not entirely satisfactory, owing to the relative slowness of the electrode response. Nevertheless the maximal rate of proton generation during ubiquinol₁ oxidation was about 1200 ng-ions of H^+ /min per mg of mitochondrial protein. It is concluded, contrarily to Chance & Mela (1967), that mitochondria exhibit a proton-translocating ubiquinol oxidase activity with a $\rightarrow\text{H}^+/\text{2e}^-$ ratio of 4.0.

Measurements of the stoichiometry of mitochondrial respiration-driven proton translocation have been made mainly by the oxidant pulse method of Mitchell & Moyle (1967) in which the pH of an anaerobic substrate-supplemented mitochondrial suspension is measured while small pulses of oxygen are added either as air-saturated solutions or as H_2O_2 . In the latter case, catalase (EC 1.11.1.6) must also be present (Chance & Mela, 1967). The mitochondrial respiratory chain reduces pulses of oxygen rapidly and completely, and the extent of the respiration-driven burst of proton translocation, measured as an acidification of the suspending medium, is not obscured by the slower return of protons back across the inner mitochondrial membrane.

Apart from the pulse-oxidant method, a pulse-reductant method may also be envisaged. Physiological substrates such as 3-hydroxybutyrate and tricarboxylic acid cycle intermediates are oxidized by mitochondria with apparent K_m values of very approximately 10^{-4} – 10^{-3} M, values that are too high for their use as pulse reductants. Palmitoyl-L-carnitine at 10 μ M is completely and quite rapidly oxidized by mitochondria, but its use would be complicated by the dual input of reducing equivalents to the respiratory chain by both NAD- and flavin-dependent de-

hydrogenases (Garland *et al.*, 1967). We have previously used ubiquinone homologues as pulse oxidants (Lawford & Garland, 1972), and in view of this and the ability of duroquinol (i.e. reduced 2,3,5,6-tetramethylbenzoquinone) to provide pulses of reducing equivalents to mitochondrial suspensions (Boveris *et al.*, 1971), we investigated the use of ubiquinol₁ (i.e. reduced ubiquinone₁) and duroquinol as pulse reductants in experiments designed to measure the ratio of protons translocated to reducing equivalents consumed by the respiratory chain, i.e. the $\rightarrow\text{H}^+/\text{2e}^-$ ratio (Mitchell, 1966). These investigations served three purposes. First, they provide indirect information by analogy on the $\rightarrow\text{H}^+/\text{2e}^-$ ratio for the oxidation of endogenous ubiquinol₁₀. This ratio would be 2.0 according to the earlier formulation of Mitchell (1966), and 4.0 according to Skulachev (1971) and Lawford & Garland (1972). Secondly, they provide a partially independent method of measuring the stoichiometry of respiration-driven proton translocation. Thirdly, the rates of quinol oxidation can be measured spectrophotometrically and there is therefore the possibility of measuring the $\rightarrow\text{H}^+/\text{2e}^-$ ratio from the initial rates of proton translocation and quinol oxidation rather than from their time-integrated extents. We find that the $\rightarrow\text{H}^+/\text{2e}^-$

ratio for mitochondrial quinol oxidation by oxygen approximates to 4.0 whether measured by time-integrated extents or initial velocities of proton translocation and quinol oxidation.

Experimental

Preparation of mitochondria and submitochondrial particles

Ox heart mitochondria were prepared by procedure 3 of Smith (1967), except that 0.25 M-sucrose was used for the final wash and resuspension. Submitochondrial particles were prepared as described by Low & Vallin (1963).

Chemicals

Antimycin A and rotenone were from Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, Surrey, U.K. Carbonyl cyanide *m*-chlorophenylhydrazine (Heytler, 1963) and valinomycin were from Calbiochem Ltd., London W.1, U.K. Duroquinone was from Ralph N. Emanuel Ltd., Wembley, Middx., U.K. Ubiquinone₁ was a generous gift from Hoffmann-LaRoche, Basel, Switzerland. The disodium salt of NADH was purchased from Boehringer Corp. (London) Ltd., London W.5, U.K. Standard solutions of HCl for calibration of electrode responses were purchased from British Drug Houses Ltd., Poole, Dorset, U.K.

Preparation of quinols

Both duroquinol and ubiquinol₁ were prepared as described by Lester *et al.* (1959) by reduction of their methanolic solutions with KBH₄ followed by acidification to about pH 3 with 0.1 M-HCl. The quinols were susceptible to autoxidation, particularly in the case of duroquinol, and they were therefore prepared only as needed and kept at 0°C. In experiments designed to measure the rate of proton translocation the pH of the quinol solution was more carefully adjusted to about pH 6.0, thereby avoiding a pH change that could arise from the quinol solution itself rather than from the mitochondrial oxidation of quinol.

Assays

Both duroquinol and ubiquinol₁ were assayed by measuring polarographically the oxygen consumed when limiting amounts of quinol were oxidized to quinone by submitochondrial particles. NADH was assayed spectrophotometrically at 340 nm, and protein was assayed by the method of Lowry *et al.* (1951) with dry bovine serum albumin as the standard (British Drug Houses Ltd.). Polarographic measurements of oxygen were made with a Clark oxygen

electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio, U.S.A.) calibrated as described by Chappell (1964).

Apparatus and procedure for the measurement of mitochondrial proton translocation

The incubation chamber, pH electrode and recording system have been described previously (Lawford & Garland, 1972) except that the overall response time was lowered to 0.6 s. In experiments designed to measure the rate rather than extent of proton translocation, the commercial pH-meter was replaced by a fast-responding high-impedance voltage follower followed by a second amplifier yielding an overall sensitivity at the output of 1.0 V/pH unit. The response time of the circuit, defined as the time, $t_{1/e}$, taken to achieve $(1 - 1/e)$ of its response to an instantaneous change at the input, where e is the base of natural logarithms, was selected with a two-pole low-pass active filter (Scott, 1966) to give values of 22, 68 and 220 ms. In practice mixing of the mitochondrial suspension and boundary layers at the electrode (Sirs, 1958) were rate limiting for the overall response time, and the filter was set at a response time of 68 ms; a faster setting did not alter the overall response time of the system, which was 0.4 s. The amplifier output was displayed on a storage oscilloscope (Model 564; Tektronic Guernsey Ltd., Channel Islands, U.K.) at a vertical amplifier sensitivity of 10 mV (equivalent to 0.01 pH unit) per cm. Experiments were started by placing about 5 mg of mitochondrial protein in 0.2 ml of 0.25 M-sucrose in the incubation chamber. The chamber was then filled with air-saturated 150 mM-KCl–3.3 mM-glycylglycine buffer, pH 7.2 at 25°C, the electrode in its plug mounted and the stirrer switched on. Rotenone (10 μ l of a 0.2 mg/ml methanolic solution) and valinomycin (1 μ l of a 0.5 mg/ml ethanolic solution) were added with micro-syringes and the pH was adjusted to 7.2 with 50 mM-KOH. Additions of quinols and inhibitors (see the Results section) were made after a 5 min delay from the onset of the experiment. In some experiments the final protein concentration was varied between 1.25 and 10.0 mg/ml. The working volume of the incubation chamber was 1.0 ml.

Spectrophotometric measurement of quinol oxidation by intact mitochondria

Although duroquinone and ubiquinone₁ display absorption maxima at 265 nm and 275 nm respectively, their oxidized-minus-reduced extinction coefficients are identical at 275 nm ($\epsilon = 12500$ litre $\cdot$ mol⁻¹ $\cdot$ cm⁻¹; Lawford & Garland, 1972). The oxidation of exogenous duroquinol and ubiquinol₁ by ox heart mitochondria was measured at 275 nm with a split-beam spectrophotometer (Haddock & Garland,

1971) equipped with a 25 W deuterium arc lamp and a stray-light filter of 3 mm thick UG5 glass (Jena Glaswerk, Schott & Gen., Mainz, W. Germany) at the entrance slit. Measurements of the extinction difference at 275 nm between test and reference cuvettes each of 1.0 cm light-path were made at 25°C in a final volume of 2.5 ml of 150 mM-KCl–20 mM-Tris–HCl, pH 7.4, containing 2 µg of rotenone, 1 mM-Tris–malonate and 0.2–0.5 mg of mitochondrial protein. Quinol oxidation was initiated by adding the quinol to the test cuvette, and recorded as an increase in extinction at 275 nm. In the absence of mitochondria, autoxidation was not detectable over the 2–3 min of the assay. The rate of quinol oxidation was proportional to the concentration of mitochondrial protein provided that it did not exceed 0.2 mg/ml. Above this limit stray light caused errors.

P/O ratios

Measurements of the ratio of ATP synthesized to atoms of oxygen utilized (P/O ratio) for the oxidation of ubiquinol₁ and duroquinol were made polarographically (Chance & Williams, 1956).

Analogue computer simulation

Analogue simulation of the effects of proton back-flow and measuring-time response on the observed kinetics of respiration-driven pH changes in the mitochondrial incubation medium were kindly carried out by Dr. J. C. Kernohan of this Department, by using a VIDAC 169 computer (Computing Techniques, Billingshurst, Sussex RH14 9RZ, U.K.).

Results

Suitability of quinols as pulse reductants of the respiratory chain for studies of proton translocation

The criteria for acceptance are identical with those required of pulse oxidants (Mitchell & Moyle, 1967; Lawford & Garland, 1972), namely, a high rate of oxidation even at low concentration, and absence of oxidoreduction pathways that by-pass parts of the normal sequence of carriers. In addition, the oxidized form of the pulse reductant must not be reduced again by endogenous substrates, for otherwise the number of reducing equivalents oxidized will be in excess of that calculated from the amount of added quinol. The oxygen-electrode recorder tracings in Fig. 1 show that pulses of both duroquinol and ubiquinol₁ are rapidly oxidized by ox heart mitochondria. The oxidation rate was approximately 95% inhibited by antimycin A and the remaining antimycin A-resistant rate was inhibited by cyanide. We have previously shown that in ox heart mitochondria the reduction of duroquinone or ubiquinone by intramitochondrial NADH is inhibited by rotenone by 53 and

89% respectively, leaving rotenone-insensitive NADH–quinone reductase activities of 15 nmol/mg per min for duroquinone and 9 nmol/min per mg for ubiquinol₁ (Lawford & Garland, 1972). Thus the rates of re-reduction of quinone by NADH are not more than about 5% of the rates of oxidation of quinol in pulse-reductant experiments with ox heart mitochondria. The experiments of Fig. 1 also demonstrate the stoichiometry of duroquinol and ubiquinol oxidation by oxygen. In each case the ratio of mol of quinol added to g-atom of oxygen was 1.0. The end-product of quinol oxidation is therefore quinone, not semiquinone, assuming that the other end-product is H₂O and not H₂O₂. This assumption is justified by the inhibitor sensitivity of the quinol oxidation.

Further information on the kinetic behaviour of quinol oxidation by ox heart mitochondria was obtained by direct spectrophotometric measurements (Fig. 2). The $V_{\max}$ of quinol oxidation obtained under these conditions would correspond to the state 3 rate (Chance & Williams, 1956), since the mitochondria are substrate-deficient until quinol is added, and state 4 is not established immediately on addition of substrate (Boveris *et al.*, 1971). The $V_{\max}$ values for the oxidation of ubiquinol₁ or duroquinol are similar (Fig. 2); they are also close to the value reported for pigeon heart mitochondria and duroquinol by Boveris *et al.* (1971). Ubiquinol₁ has a K_m for oxidation that is nearly fourfold lower than that of duroquinol, and is therefore the preferred substrate for pulse-reductant usage if speed of reaction is the predominant consideration.

→H⁺/2e⁻ ratios for the mitochondrial oxidation of quinols by oxygen

The pH changes occurring on addition of pulses of duroquinol or ubiquinol₁ to aerobic and substrate-deficient mitochondria are illustrated in Fig. 3. In either case there was a fast phase of acidification which was completed within 3–4 s and which was followed by an exponential decay of the pH change with a $t_{\frac{1}{2}}$ of approx. 10 s. The rate of the acidification phase was not less than 4 ng-ions of H⁺/s per mg of protein, and this value is limited by the response-time of the mixing, amplifying and recording system. The pH does not relax back to its original value in these experiments, and this is due to acidification by the quinol solution itself, and not to quinol oxidation; the shift in pH on addition of quinol to mitochondria after addition of the respiratory inhibitors antimycin A and cyanide demonstrates this point (Fig. 3). If 0.1 µM-carbonyl cyanide *m*-chlorophenylhydrazone was added at the peak of the acidification phase after a pulse of quinol, then the respiration-driven pH change collapsed within 1–2 s; subsequent additions of quinol caused only the pH shift due to the acidity of the quinol

Fig. 1. Polarographic recordings of oxygen uptake by ox heart mitochondria

The experiments were done in a water-jacketed chamber at 30°C, by using a Clark electrode to measure oxygen consumption. In (a) ox heart mitochondria (1.25 mg of protein) were added to 3.0 ml of 150 mM-KCl–20 mM-Tris–HCl buffer, pH 7.4, and in (b) ox heart mitochondria (1.6 mg of protein) were added to 2.5 ml of the same buffer. Further additions in both (a) and (b) were: K_2HPO_4 (6.0 μ mol), rotenone (Rot., 2 μ g), succinate (Succ., 3.0 μ mol), malonate (Mal., 6.0 μ mol), antimycin A (AA, 0.25 μ g) and KCN (2.5 μ mol). In (a) ubiquinol₁ (QH₂, 269 nmol) and in (b) duroquinol (DQH₂, 360 nmol) were added. For purposes of comparison, the rates of succinate and quinol oxidation (nmol/min per mg of protein) are indicated in each experiment.

Fig. 2. Double-reciprocal plot of exogenous quinol concentration and initial rate of quinol oxidation in intact ox heart mitochondria

The rate of oxidation of added ubiquinol₁ (○) and duroquinol (Δ) in intact ox heart mitochondria was measured spectrophotometrically at 275 nm at 25°C as described in the Experimental section. The apparent K_m for duroquinol is 28 μ M and for ubiquinol₁ is 8 μ M and the V_{max} (nmol/min per mg of protein) is 260 and 280 for duroquinol and ubiquinol₁ respectively.

solution. When measuring the extent of proton translocation due to quinol oxidation, correction was made for the pH change due to the quinol solution. If high protein concentrations (10 mg/ml) and prolonged incubations are used, the slow residual rate of respiration of endogenous substrate may cause the suspension to become anaerobic. This is readily recognized by the appearance of acidification in response to additions of air-saturated KCl (Mitchell & Moyle, 1967). The extent of the acidification due to quinol oxidation was proportional to the amount of quinol added over the range 4–24 μ M when 5 mg of mitochondrial protein was used. The average observed $\rightarrow H^+/2e^-$ ratio (i.e. that calculated from the peak of acidification and uncorrected for decay) was 3.62 for ubiquinol₁ and 2.98 for duroquinol (Table 1).

Correction of the observed $\rightarrow H^+/2e^-$ ratios

The observed peak of acidification in both pulse-reductant and pulse-oxidant experiments is underestimated by an error that arises from the flow of protons back across the inner mitochondrial membrane during the non-instantaneous burst of respiration. This error is worsened by prolongation of the

Table 1. Observed $\rightarrow H^+/2e^-$ quotient with quinol as substrate and oxygen as terminal respiratory acceptor and values of $\rightarrow H^+/2e^-$ that have been corrected for error in ΔH^+ measurement due to proton back-flow

Details of experimental conditions for pH measurements are given in the legend to Fig. 3. The concentration of quinol was $8\mu M$ and that of mitochondrial protein was 5mg/ml. Values for $\rightarrow H^+/2e^-$ were corrected by the method outlined in the Results section. The values of $\rightarrow H^+/2e^-$ quotient are presented as the mean $\pm$ s.e.m. with the number of observations in parentheses.

Substrate	V_{max} . (nmol/min per mg of protein)	K_m (μM)	$\rightarrow H^+/2e^-$	
			Observed	Corrected
Ubiquinol ₁	280	8	3.62 ± 0.14 (12)	4.01 ± 0.16
Duroquinol	260	28	2.98 ± 0.18 (8)	3.86 ± 0.22

Fig. 3. pH recordings of proton translocation by ox heart mitochondria with ubiquinol₁ or duroquinol as substrate and oxygen as terminal respiratory acceptor

The apparatus, experimental procedure and conditions are outlined in detail in the Experimental section. An upward deflexion corresponds to an acidification of the suspending medium. In (a) ox heart mitochondria (6mg of protein) were added to air-saturated 150mM-KCl-3.3mM-glycylglycine buffer, pH7.2 at 25°C; the total volume of the aerobic mitochondrial suspension was 1.0ml. Rotenone (2 μ g) and valinomycin (0.5 μ g) were added immediately after the incubation chamber was sealed with the electrode plug; the pH was adjusted to pH7.2 with 50mM-KOH or 50mM-HCl. After 5min for equilibration, additions were made as indicated by the arrows: air-saturated 150mM-KCl (ng-atoms of oxygen added are indicated); amount (nmol) of ubiquinol₁ (QH₂) is indicated; methanol (MeOH, 10 μ l). The system was calibrated with HCl (10nmol). During the time-interval shown, antimycin A (0.6 μ g) was added. In (b) ox heart mitochondria (6mg of protein) were added to a total volume of 1.0ml of air-saturated 150mM-KCl-3.3mM-glycylglycine buffer, pH7.2 containing rotenone (2 μ g) and valinomycin (0.5 μ g). The amount (nmol) of duroquinol (DQH₂) added is indicated. Further additions were: carbonyl cyanide *m*-chlorophenylhydrazone (CCCP, 0.5nmol) and during the time-interval shown antimycin A (AA, 0.6 μ g) and KCN (0.5 μ mol) were added and the pH was adjusted to 7.2 with 50mM-HCl. The observed $\rightarrow H^+/2e^-$ ratio is indicated beside each trace.

respiratory phase and/or a decrease of the $t_{\frac{1}{2}}$ for the exponential decay of the acidification peak. Pulses of oxygen are very rapidly consumed, and Mitchell & Moyle (1967) made correction for the slight underestimate in their observed $\rightarrow H^+/2e^-$ ratios by making semi-logarithmic plots of the decay phase and extrapolating them back to the moment of adding oxygen.

The decay rates in our experiments with ox heart mitochondria were appreciably faster than those observed with rat liver mitochondria (Mitchell & Moyle, 1967), and it became apparent that correction by semi-logarithmic plotting was open to considerable subjective error when the decay rate was high and there was doubt as to the exact point in time to which

extrapolation should be made. This doubt would be removed if the burst of respiratory activity were virtually instantaneous, but is increased if the burst is prolonged, as with a pulse-reductant or oxidant with a rather high K_m , such as duroquinol or duroquinone.

To provide a more accurate correction of the observed $\rightarrow H^+/2e^-$ ratios an analogue-computer simulation was made in collaboration with Dr. J. C. Kernohan of this Department. The rationale behind this model was as follows: proton translocation associated with quinol oxidation can be expressed as the sum (eqn. 3) of the two part reactions of quinol oxidation (eqn. 1) and proton translocation (eqn. 2), where 'in' and 'out' refer to inside and outside the inner mitochondrial membrane.

Sum:

It is assumed that reactions (1) and (2) are indeed coupled to yield reaction (3), that in the presence of excess of oxygen the reaction is zero order with respect to oxygen, and that Michaelis-Menten kinetics (Michaelis & Menten, 1913) govern the oxidation of

quinol (see Fig. 2). If S is the quinol concentration then:

$$\frac{dS}{dt} = \frac{-V_{max} \cdot S}{S + K_m} \quad (4)$$

and

$$n \cdot \frac{dS}{dt} = \frac{d[H^+]}{dt} \quad (5)$$

V_{max} is expressed as $\text{nmol} \cdot \text{s}^{-1}$ and therefore incorporates the mitochondrial protein concentration. Eqns. (4) and (5) were used to simulate the time-course of the proton-translocating quinol oxidation. The numerical value assigned to n does not affect the analysis, provided that it is greater than zero. Reaction (6):

is the back-flow of protons proceeding as an exponential decay with a measured half-time of $t_{\frac{1}{2}}$ s, and is governed by eqn. (7):

$$\frac{-d(\Delta H^+)}{dt} = K\Delta H^+ \quad (7)$$

where ΔH^+ is the extent of respiration-driven acidification observed with the electrode and K is the rate constant for the decay of ΔH^+ calculated from the observed $t_{\frac{1}{2}}$.

Eqns. (4), (5) and (7) were set up on the analogue

Fig. 4. Recordings of computer simulations of proton translocation coupled to ubiquinol₁ and duroquinol oxidation in ox heart mitochondria

Three separate computer-simulated experiments are shown. The model on which these simulations were based is described in detail in the Results section. The first two experiments represent $\rightarrow H^+$ coupled to ubiquinol₁ oxidation by using 5 mg and 1.5 mg of mitochondrial protein respectively. Values of other kinetic parameters substituted in the equations given in the Results section were: $K_m = 8 \mu\text{M}$; $S_0 = 8.6 \mu\text{M}$; and $t_{\frac{1}{2}}$ (decay) = 10s. The third experiment (to the right) represents duroquinol oxidation with 5 mg of mitochondrial protein; other parameters used were: $K_m = 28 \mu\text{M}$, $S_0 = 9.0 \mu\text{M}$; $t_{\frac{1}{2}}$ (decay) = 10s. Lines (a), (d), (g) represent the acidification of the medium in the absence of proton back-flow across the membrane, lines (b), (e), (h) represent the case where the proton pulse decays exponentially, and lines (c), (f), (i) are with a 0.43 s response time superimposed on the conditions described for (b), (e) and (h).

computer to yield ΔH^+ as a function of time, and a further rate constant was sometimes incorporated to simulate the response time of the pH-measuring and pen-recording system. Fig. 4 shows the simulated time-course of $[H^+]_{out}$ in response to pulses of quinol. The first three curves (Fig. 4a,b,c) show the time-course during ubiquinol oxidation (a) in the absence of proton back-flow, when the observed change in $[H^+]_{out}$ is equal to the true extent of proton translocation, (b) with a $t_{\frac{1}{2}}$ for the decay of 10s, and (c) with a superimposed response time of 0.43s. These curves are for 5mg of mitochondrial protein. It is clear that even with a $t_{\frac{1}{2}}$ of as little as 10s the observed extent of proton translocation is only 9% in error. Replacement of an ideal measuring system of instant response with one with slower response increased the error to 11%, although the error in the observed initial velocity of proton translocation is half the true value. The next three curves (Fig. 4d,e,f) correspond to the first three but at a lower V_{max} , set by the use of 1.5mg of mitochondrial protein; the error in the observed extent of proton translocation has risen to 23%. The final three curves (Fig. 4g,h,i) simulate proton translocation associated with duroquinol oxidation. The K_m for duroquinol is relatively high, the respiratory phase is prolonged, and the error in the observed extent of H^+ translocation is 20% at 5.0mg of mitochondrial protein.

In our mitochondrial experiments the observed extent of proton translocation was proportional to the amount of quinol added, and the major source of variable error lay in different protein concentrations. This is apparent from the computer simulation studies shown in Fig. 5, where the percentage error in the observed extent of proton translocation is plotted as a function of the mitochondrial protein used for both ubiquinol₁ and duroquinol oxidation. These curves were used to correct the observed $\rightarrow H^+/2e^-$ values listed in Table 1. The average $\rightarrow H^+/2e^-$ ratio for ubiquinol oxidation rose on correction from 3.62 to 4.01, and from 2.98 to 3.86 for duroquinol oxidation.

To substantiate the validity of the use of the correction curves of Fig. 5 we carried out a number of experiments where the mitochondrial protein concentration was varied and all other conditions, including the batch of mitochondria, were kept constant. The pH recordings in Fig. 6 show how the observed $\rightarrow H^+/2e^-$ ratio fell with diminishing protein concentration or the substitution of duroquinol for ubiquinol₁. If it is assumed that the true $\rightarrow H^+/2e^-$ ratio is 4.0 then the values observed in Fig. 6 agree closely with those predicted from Fig. 5.

Rates of proton translocation

A rapidly responding meter was constructed which, with the simple mixing system in use (Lawford &

Garland, 1972), gave a response time of 0.4s on injection of acid into the reaction chamber. Fig. 7 shows oscilloscope tracings of the pH changes occurring in the first few seconds after addition of a pulse of neutralized ubiquinol₁ to aerobic rotenone-treated ox heart mitochondria. The concentration of ubiquinol₁ used was $8.6\mu M$ and would be oxidized at a

Fig. 5. Plot of percentage error in pH measurements against the amount of mitochondria used in determining proton translocation coupled to ubiquinol₁ and duroquinol oxidation

Values for percentage error were taken from analogue-computer simulations as shown in Fig. 4, with the following parameters: for duroquinol, $K_m = 28\mu M$; $S_0 = 9.0\mu M$, $t_{\frac{1}{2}}$ (decay) = 10s, $V_{max} = 260$ nmol of duroquinol oxidized/min per mg of protein (Fig. 2) and a superimposed response time of 0.6s, which is appropriate for the commercial pH-meter and pen-recorder used for measurements of $\rightarrow H^+/2e^-$ ratios (see the Experimental section). For ubiquinol₁, $K_m = 8\mu M$; $S_0 = 8.6\mu M$; $V_{max} = 280$ nmol of ubiquinol₁ oxidized/min per mg of protein, and $t_{\frac{1}{2}}$ (decay), and the response time as for duroquinol. ○, Ubiquinol₁ oxidation; Δ, duroquinol oxidation.

Fig. 6. Effect of mitochondrial protein concentration on value of $\rightarrow H^+/2e^-$ for ubiquinol₁ and duroquinol oxidation

In (a) ubiquinol₁ was used as substrate ($9.4 \mu\text{M}$) with 5 mg, 2.5 mg and 1.25 mg of mitochondrial protein respectively, in experiments shown left to right. Observed values of $\rightarrow H^+/2e^-$ are respectively 3.80, 3.40 and 2.95; values of $\rightarrow H^+/2e^-$ corrected for proton decay are 4.16, 4.08 and 3.96. In (b) duroquinol was used as substrate ($14.8 \mu\text{M}$) with 5, 2.5 and 1.25 mg of mitochondrial protein respectively in experiments shown left to right. Observed values of $\rightarrow H^+/2e^-$ are 3.10, 2.55 and 1.66; corrected values of $\rightarrow H^+/2e^-$ are 3.90, 3.65 and 3.50.

rate of about 145 nmol/min per mg of protein (Fig. 2). The observed rate of acidification 0.5 s after initiation was 550 ng-ions of H^+ /min per mg of protein, which gives a $\rightarrow H^+/2e^-$ ratio of 3.8. At a near-saturating concentration of ubiquinol₁ the rate of proton translocation was 1200 ng-ions/min per mg, which is 4.25 times the comparable rate of ubiquinol oxidation. More extensive studies were not performed owing to the marginally acceptable response time of the pH-measuring system. Ideally one would like to obtain simultaneous glass-electrode measurements of pH changes and spectrophotometric measurement of quinol oxidation, by using a stopped-flow apparatus to overcome boundary-layer effects (Sirs, 1958).

P/O ratios for quinol oxidation

The polarographically determined P/O ratios for duroquinol (0.42 mM) and ubiquinol₁ (0.38 mM) oxidation were 1.75 and 1.90 respectively, and therefore very similar to the value for succinate.

Discussion

It is a well established experimental fact that under appropriate conditions mitochondria exhibit a rapid and extensive respiration-driven proton translocation (Mitchell & Moyle, 1967; Packer & Utsumi, 1969; Peniston *et al.*, 1971; Papa *et al.*, 1972). Earlier reservations (e.g. Chance & Mela, 1967) appear to have been withdrawn (e.g. Papa *et al.*, 1972), and the slow rates of proton translocation measured spectrophotometrically (1.2 ng-ions of H^+ /min per mg of protein for oxygen-pulsed ox heart submitochondrial particles; Chance & Mela, 1967) have now been replaced by electrode measurements with rates that are over 1000-fold faster (3180 ng-ions of H^+ /min per mg of protein; Papa *et al.*, 1972). Measurements of the stoichiometry of respiration-driven proton translocation are clearly of importance in determining the underlying mechanism. Measurements related to ion movements during ion-gradient-driven ATP synthesis (Cockrell *et al.*, 1967; Rossi & Azzone, 1970) do not

Fig. 7. Oscilloscope tracings of pH changes occurring in the first few seconds after addition of ubiquinol₁ to aerobic mitochondria

The laboratory-constructed faster-responding pH meter was used with a storage oscilloscope, and the response time of this system is 0.4 s provided that the manual injection of ubiquinol₁ (or calibrating acid solution) from a microsyringe into the incubation is not made slowly. The upper two traces show acidification of the medium following the injection of 8.6 nmol of ubiquinol₁ into a suspension of aerobic, rotenone-treated mitochondria (5 mg of protein per ml). The pH of the suspending medium returned to the original value within 1 min of the addition of ubiquinol₁. The initial small and temporary alkalinisation is not reproducible, occurs with injections of acid or ubiquinol₁, does not occur when slow injection is made, and is probably an artifact of mechanical origin. The lowest trace shows the response to a more slowly injected calibrating pulse of 5 mM-HCl (20 ng-ions of H⁺). Rapid injections of acid show a response time of 0.4 s.

shed any direct light on the stoichiometry of respiration-driven proton translocation, but instead measure the reversibility of ATP-driven ion transport.

The experiments reported by us demonstrate the feasibility of the pulse-reductant method for studying respiration-driven proton translocation coupled to ubiquinol₁ and duroquinol oxidation. The $\rightarrow\text{H}^+/\text{2e}^-$ ratios are close to 4.0, as is to be expected for a substrate that communicates with the respiratory chain between the rotenone- and antimycin A-sensitive sites and is oxidized with a P/O ratio of 2.0. Although the use of these quinols may tell us much of their own behaviour, it is not clear as to how far they

behave similarly to endogenous ubiquinol₁₀. The studies of Boveris *et al.* (1971) indicated that endogenous ubiquinone₁₀ was rapidly reduced and oxidized during cycles of duroquinol oxidation. However, Kröger (1972) and Kröger *et al.* (1973) did not consider that ubiquinone₁₀ was an obligatory intermediate for duroquinol oxidation.

According to the chemiosmotic hypothesis (Mitchell, 1966) there should be a strict stoichiometry between the rate of respiration-driven proton translocation and the rate of respiration. Chance & Mela (1967) claimed that in oxygen-pulsed submitochondrial particles the rate of ubiquinol₁₀ oxidation was 1000-fold faster than proton translocation, and concluded that respiration-driven proton translocation as envisaged by Mitchell (1966) could not be supported. Our own measurements of proton translocation driven by ubiquinol₁₀ oxidation in whole mitochondria showed a V_{max} for oxidation of 280 nmol/min per mg of protein and for proton translocation of 1200 ng-ions of H⁺/min per mg of protein, yielding a $\rightarrow\text{H}^+/\text{2e}^-$ ratio of 4.3. There are obvious technical limitations to these results; the oxidation rates are steady-state rates at low protein concentration, and the transient proton-translocation rates are at the limit of the measuring response of the apparatus and at higher protein concentrations. Nevertheless these experiments indicate the advantage of the use of ubiquinol₁ and duroquinol as pulse reductants, and the $\rightarrow\text{H}^+/\text{2e}^-$ values obtained for both extent and rate provide further evidence for a strict coupling of proton translocation to mitochondrial respiration. The particular advantages of ubiquinol₁ and duroquinol are (1) high rates of mitochondrial oxidation, (2) freedom from autooxidation or other respiratory-chain by-passes, (3) sufficiently low K_m values for use as pulse reductants, (4) a spectroscopically measurable rate of oxidation that may permit simultaneous measurements of the rates of proton translocation and oxidation, (5) diffusion to the respiratory chain that is not dependent on a translocase, and (6) absence of charge at physiological pH values and inability therefore to participate in ion movements across the membrane. Further, these quinols may be well suited for studies of proton-translocating respiration in micro-organisms.

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