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Biochemistry

Proton translocation stoichiometry of cytochrome oxidase: Use of a fast-responding oxygen electrode

(cytochrome *c*/vectorial H^+ movements/electron transport)

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ABSTRACT **The mechanistic stoichiometry of vectorial H^+ ejection coupled to electron transport from added ferrocytochrome *c* to oxygen by the cytochrome oxidase (EC 1.9.3.1) of rat liver mitoplasts was determined from measurements of the initial rates of electron flow and H^+ ejection in the presence of K^+ (with valinomycin). Three different methods of measuring electron flow were used: (*a*) dual-wavelength spectrophotometry of ferrocytochrome *c* oxidation, (*b*) uptake of scalar H^+ for the reduction of O_2 in the presence of a protonophore, and (*c*) a fast-responding membraneless oxygen electrode. The reliability of the rate measurements was first established against the known stoichiometry of the scalar reaction of cytochrome oxidase (2ferrocytochrome *c* + $2H^+$ + $\frac{1}{2}O_2$ → 2ferricytochrome *c* + H_2O) in the presence of excess protonophore. With all three methods the directly observed vectorial H^+/O ejection ratios in the presence of K^+ + valinomycin significantly exceeded 3.0. However, because the rate of backflow of the ejected H^+ into the mitoplasts is very high and increases with the increasing ΔpH generated across the membrane, there is a very rapid decline in the observed H^+/O ratio from the beginning of the reaction. Kinetic analysis of ferrocytochrome *c* oxidation by the mitoplasts, carried out with a fast-responding membraneless oxygen electrode, showed the reaction to be first order in O_2 and allowed accurate extrapolation of the rates of O_2 uptake and H^+ ejection to zero time. At this point, at which there is zero ΔpH across the membrane, the H^+/O ejection ratio of the cytochrome oxidase reaction, obtained from the rates at zero time, is close to 4.0.**

The mechanistic stoichiometry of vectorial H^+ translocation by cytochrome oxidase (ferrocytochrome *c*:oxygen oxidoreductase, EC 1.9.3.1) is crucial in the intensive current efforts to define the structure and mechanism of this complex enzyme. However, there has been substantial disagreement regarding the vectorial H^+/O ejection ratio of the cytochrome oxidase reaction. We (1–3) and others (4, 5) have reported data consistent with H^+/O ratios close to 4, whereas several groups have reported values near 2 (6–12), and two groups claim that no vectorial H^+ ejection is directly coupled to cytochrome oxidase (13, 14). These discrepancies require resolution with appropriately validated methods.

We report here determination of the mechanistic H^+/O ejection ratio for cytochrome oxidase with ferrocytochrome *c* (C^{2+}) as electron donor instead of nonbiological reductants which require undesirable assumptions or corrections. Rat liver mitoplasts were used to eliminate the mitochondrial outer membrane as permeability barrier to added C^{2+}. Three methods, whose reliability was first established against a reaction of known stoichiometry, were used to monitor electron flow through cytochrome oxidase: (*i*) dual-wavelength spectrophotometry of ferricytochrome *c* (C^{3+}) formation, (*ii*) glass electrode measurements of the scalar uptake of H^+ required for the reduction of oxygen to H_2O, and (*iii*) measurement of oxygen uptake with a fast-responding membraneless oxygen electrode. The initial rate measurements used gave directly observed vectorial H^+/O ejection ratios for the cytochrome oxidase reaction exceeding 3. When extrapolated to the condition of level flow—i.e., zero transmembrane pH—the H^+/O ratio closely approached 4, in confirmation of our earlier data (1–3).

EXPERIMENTAL DETAILS

The rat liver mitoplasts (15) had respiratory control ratios of 4–6 with carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) present. The optical measurements (as in Figs. 1 and 2) were carried out at 20°–22°C in a round 1.0-cm glass cell in an Aminco DW-2 spectrophotometer. The aerobic reaction medium (3.0 ml) was stirred at 2,000 rpm by vanes on an upright, cylindrical, Teflon-coated, magnetic rotor, milled to fit the round-bottomed cell with 0.5-mm lateral clearance and driven by an air turbine. A combination pH electrode (Beckman 39505) and a Teflon port for additions were fixed in a plastic stopper. The sensitive portion of the glass electrode was placed as close to the optical path as possible, positioned to prevent vortex formation at the high stirring speed. Patient adjustment of the cell and electrode geometry minimized the mixing time and mechanical noise. Outputs of the spectrophotometer and glass electrode were amplified and fed into a Soltec (Sun Valley, CA) model 330 multichannel recorder run at a chart speed of 120 cm/min.

Formation of C^{3+} was monitored at 535.3–505.0 nm, with full-scale deflection given by $\Delta A = 0.75$. Under these conditions, only the formation of C^{3+} gave a signal. For the experiments in Figs. 1 and 2, C^{3+} (Sigma, type VI) was reduced with hydrosulfite and the C^{2+} was recovered from a Sephadex G-25 column, followed by dialysis under N_2 and lyophilization. The C^{2+} stock solution, which usually contained some C^{3+}, was made up to 7–8 mM and its surface was swept with wet N_2. Its pH was precisely adjusted so that, when it was mixed with the rest of the system, including the mitochondria and 1.0 mM neutral cyanide to inhibit cytochrome oxidase, no scalar pH change resulted. The optical response to C^{3+} formation was calibrated with successive additions of 40.0 nmol of ferricyanide to the complete system in the presence of cyanide. H^+ changes were calibrated against internal standards approximating the actual range of H^+ uptake or ejection observed.

In the experiments of Figs. 3 and 5, oxygen uptake was measured with fast-responding O_2 electrodes modified from those described in refs. 16 and 17. Their 90% response times, monitored with an oscillograph, could be made as short as 10 ms.

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Abbreviations: C^{2+}, ferrocytochrome *c*; C^{3+}, ferricytochrome *c*; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

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For these experiments the electrodes had a response time of 200 ms, kinetically matched to the response time of the pH electrode. The measurements were carried out in a thermostatted closed chamber with essentially no gas phase, resembling that described in ref. 18. The stirring rate and chamber geometry were carefully adjusted to minimize mixing time and noise. A common reference cell was used, provided by the combination pH electrode. The slight interference in the response of the pH electrode caused by the current generated by the O_2 electrode was eliminated by precise compensation with an external bucking circuit. The electrode signals were suitably amplified and fed into a Soltec model 330 recorder. Data processing was carried out with a Hewlett-Packard 9872C digitizer and plotter, 6940B multiprogrammer, and 9845B computer.

RESULTS

Validation of Spectrophotometric Measurement of C^{2+} Oxidation and H^+ Uptake Against the Scalar Cytochrome Oxidase Reaction. Reliability of the spectrophotometric measurements of electron flow and electrode measurements of H^+ changes was first determined under the precise conditions used in the H^+/O ejection ratio experiments, by making use of the fact that, in the presence of excess protonophore (FCCP), the scalar cytochrome oxidase reaction proceeds according to the equation

$$2\text{ferrocytochrome } c + 2H^+ + \tfrac{1}{2}O_2 \rightarrow 2\text{ferricytochrome } c + H_2O \quad [1]$$

in which the H^+/e^- uptake ratio is obligatorily 1.0 (H^+/O uptake ratio = 2.0). Fig. 1 shows traces of a typical experiment carried out under such conditions. After aerobic preincubation of the mitoplasts in the test medium with excess FCCP, as well as antimycin A and rotenone to inhibit electron flow from endogenous substrates, the cytochrome oxidase reaction was initiated by rapid injection and mixing of C^{2+}. The initial rates of H^+ uptake (1,310 nmol/min·mg) and C^{2+} oxidation (1,350 nmol/min·mg), obtained from the slopes of the tangents (Fig. 1, dashed lines), give the scalar H^+/e^- ratio = 1,310/1,350 = 0.97, very close to the theoretical 1.0 for Eq. 1. In the presence of cyanide, essentially no oxidation of C^{2+} or uptake of H^+ took place. Such measurements in the presence of excess FCCP always gave H^+/e^- uptake ratios very close to 1.0, thus validating the reliability of the rate measurements.

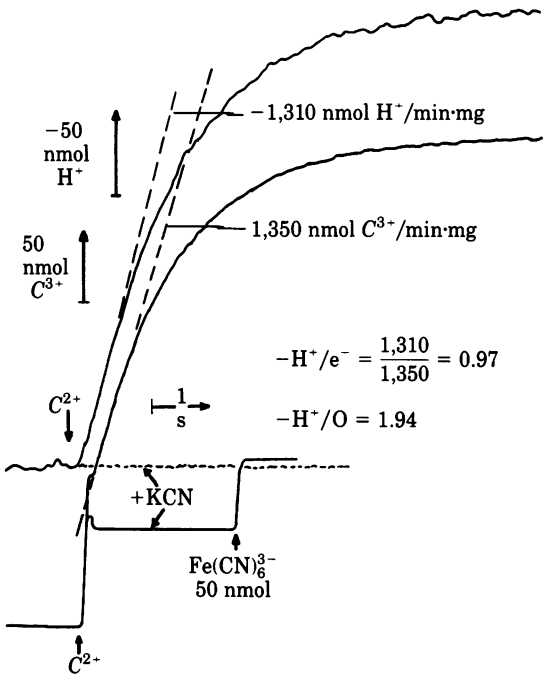

FIG. 1. Validation of spectrophotometric C^{3+} measurements against the scalar cytochrome oxidase reaction. The test system (3.0 ml) and preincubation details were exactly as in Fig. 2 except that 18 μM FCCP was present rather than valinomycin. The reaction was started by injection of C^{2+}. The instantaneous increase in the absorbance on mixing is due to the presence of some C^{3+}. The initial rates of H^+ and O_2 uptake were obtained from the slopes of the dashed tangents. No H^+ changes occurred in the absence of C^{2+}; in the presence of KCN there were no changes in either C^{2+} or H^+. Also shown is the effect of a single 40-nmol addition of ferricyanide for calibration of the optical response.

Vectorial H^+/O Ejection Ratio from Spectrophotometric Experiments. Fig. 2 shows the traces from a comparable experiment in which the vectorial H^+/e^- ejection ratio of the cytochrome oxidase reaction was determined by using the rate methods validated above. The test medium was as in Fig. 1, but FCCP was replaced with valinomycin to allow K^+ to enter in exchange for the ejected H^+. Oxidation of the added C^{2+} was accompanied by H^+ ejection, which rapidly approached a peak, followed by net H^+ backflow. The initial rate of H^+ ejection (2,100 nmol/min·mg) and of C^{2+} oxidation (1,310 nmol/min·mg) give a H^+/e^- ejection ratio of 1.63—i.e., a H^+/O ratio of 3.26. There was no significant oxygen consumption or H^+ output when C^{2+} was omitted or when cyanide was present, nor was there any detectable reduction of added C^{3+} by endogenous substrates over the short time intervals used. Reuptake of the ejected H^+ through H^+ leaks continued after the

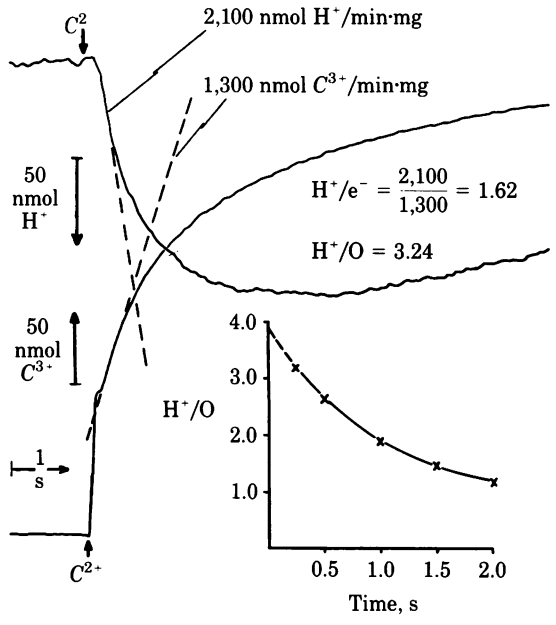

FIG. 2. The vectorial H^+/O ejection ratio of the cytochrome oxidase reaction measured spectrophotometrically. The test system (3.0 ml) contained 250 mM sucrose, 40 mM KCl, 1.5 mM K Hepes buffer (pH 7.05), 2 μM rotenone, 0.1 nmol of antimycin A per mg of protein, 1 μg of oligomycin per mg, 0.2 mM EGTA, 1.0 mg of defatted bovine serum albumin, and mitoplasts (6.0 mg of protein). After an initial 30-s incubation the pH was lowered to 6.2 by addition of HCl to allow uptake of H^+ and P_i^- into the matrix via the H^+/P_i^- symporter; 90 s later, mersalyl (40 nmol/mg) was added to prevent subsequent influx or efflux of H^+ and P_i^- and the system was back-titrated to pH 7.05. The preloading of the matrix with P_i at pH 6.2 significantly augmented the internal buffering capacity. After addition of 0.8 nmol of valinomycin per mg, the reaction was started by injection of C^{2+}; the rates of H^+ ejection and C^{3+} formation were then monitored. The vectorial H^+/O rate ratio was obtained from the tangents shown (dashed lines).

added C^{2+} was completely oxidized until the H^+ trace attained a stable end value corresponding to net uptake of exactly 1.0 H^+ per C^{3+} consumed, as required by the scalar Eq. 1.

The *Inset* in Fig. 2 shows that the observed H^+/O ejection ratio declined very rapidly with time to about 2.0 at 1.0 s and to 1.2 at 2.0 s, presumably because of the rapidly increasing rate of backflow of the ejected H^+ as ΔpH across the membrane increased during electron transport. The mechanistic H^+/O ejection ratio for the cytochrome oxidase reaction thus can be observed only very early in the reaction when ΔpH is near zero.

H^+/O Ejection Ratios from Two Successive H^+ Rate Measurements. The traces in Figs. 1 and 2, taken from two successive experiments on the same mitoplast preparation, show that the initial rate of oxidation of C^{2+} in the presence of FCCP (1,350 nmol/min·mg, Fig. 1) and the initial rate in the presence of valinomycin (1,310 nmol/min·mg, Fig. 2) are equal within experimental error, as expected if electron transport by mitoplasts is not limited by the rate of ionophore-induced H^+ or K^+ movements across the membrane. This equality provides a second way of determining the H^+/O ejection ratio of the cytochrome oxidase reaction, in which the scalar uptake of H^+ is used as a measure of the rate of electron flow according to the stoichiometry of Eq. 1. Under these conditions the vectorial H^+/e^- ejection ratio of the cytochrome oxidase reaction is given simply by

$$H^+/e^- = \frac{\text{initial rate of } H^+ \text{ ejection in the presence of valinomycin}}{\text{initial rate of } H^+ \text{ uptake in the presence of FCCP}}.$$

Two successive H^+ rate measurements, one with valinomycin (H^+ ejection) and the other with FCCP (H^+ uptake) thus suffice to determine the H^+/e^- ejection ratio. The H^+/e^- ejection ratio so obtained from the data in Figs. 1 and 2 was 2,100/1,310 = 1.60, equivalent to a H^+/O ejection ratio of 3.20. In a series of 19 such paired H^+ rate measurements under conditions of nearly equal electron flow rates, the vectorial H^+/O ejection ratios were in the range 2.90–3.92, with a mean (± SD) of 3.22 ± 0.28. Because the two successive H^+ rate measurements were made with the same glass electrode, errors caused by differences in instrument response times for the electron flow and H^+ measurements are eliminated.

Validation of Kinetic Measurements with a Fast-Responding Membraneless Oxygen Electrode. Because the rate of oxidation of C^{2+} declines continuously with time, initial rates of electron flow (and also H^+ movements) obtained from the slopes of hand-fitted tangents, as in Figs. 1 and 2, tend to be underestimated and in any case are prone to subjective error. More detailed kinetic analysis of the rate of C^{2+} oxidation in such systems was therefore carried out with a fast-responding membraneless O_2 electrode (16, 17). So long as O_2 uptake is relatively slow, as was true in our earlier studies (cf. ref. 19), its rate can be measured accurately with the Clark electrode, especially if provided with a stretched ultrasensitive membrane, as had been ascertained by appropriate calibration experiments (3). However, the response of Clark electrodes is much too slow when the rates of oxygen consumption are extremely high and nonlinear as in the experiments in Figs. 1 and 2.

The membraneless oxygen electrode used in the following experiments has a much shorter response time. To establish its reliability and its kinetic compatibility with the pH electrode, simultaneous measurements were made of the rate of O_2 uptake and the scalar uptake of H^+ when the oxidation of C^{2+} was taking place in the presence of a large excess of the protonophore FCCP—i.e., according to the scalar Eq. 1, for which the theoretical H^+/O uptake ratio is 2.0. Fig. 3 shows the H^+ and O_2

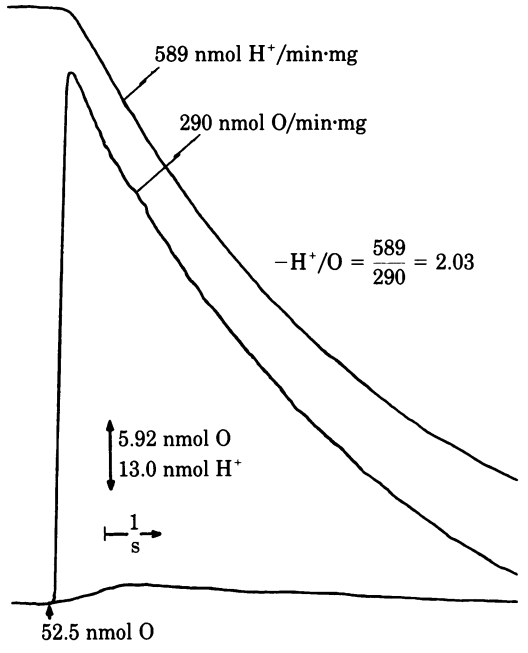

FIG. 3. Validation of the membraneless oxygen electrode measurements against the scalar cytochrome oxidase reaction. The medium contained 200 mM sucrose, 50 mM KCl, and 1.5 mM K Hepes (pH 7.05). The O_2 content of the medium was first reduced to about 20% of air saturation by bubbling with wet N_2. The reaction cell was closed and 2 μM rotenone, 20 μM FCCP, 0.5 mM succinate, 40 nmol of *N*-ethylmaleimide per mg, 200 nmol of C^{3+} and 2.2 mg of mitoplast protein were introduced in small volumes through a long, narrow port in the stopper of the cell. The system (total, 1.35 ml; t = 20°C) was then incubated under N_2 until the dissolved O_2 was completely consumed and the C^{3+} was totally reduced *in situ* by the added succinate, as monitored by the coupled H^+ ejection. Antimycin A (0.1 nmol/mg) was then added to prevent any further electron flow from the remaining succinate to ferricytochrome *c*. To initiate the cytochrome oxidase reaction, oxygen (52.5 nmol of O) was added to the anaerobic system in the form of 100 μl of air-saturated (20°C) medium. Oxygen consumption and H^+ uptake were monitored. The solubility of oxygen at 20°C in the medium was 525 nmol of O per ml. The initial rates of O_2 and H^+ uptake shown were obtained from the traces as described in the text and shown in Fig. 4. The addition of antimycin A inhibited re-reduction of C^{3+} to less than 0.5% of the rate of C^{2+} oxidation. The O_2 trace at the bottom was obtained with a Clark electrode and shows its inability to record fast changes in O_2 concentration.

traces recorded from such an experiment. The reaction was started by injection of a known amount of dissolved oxygen into the anaerobic reaction system, which already contained excess C^{2+}. The changes in concentration of O_2 at 0.2-s intervals were taken from the traces and converted into digital form, and the rates of O_2 uptake at successive 0.2-s intervals were computed. Tests of the data against various rate laws showed the reaction to be first order in O_2 up to 70% of its course. Fig. 4 shows the semilogarithmic plot of the rate of O_2 uptake vs. time; the true zero time was established from the precisely known amount of O_2 added. The rates of H^+ uptake at 0.2-s intervals were treated in the same manner and also yielded a linear semilogarithmic plot. The intercepts at zero time gave extrapolated initial rates of 589 nmol/min·mg for H^+ uptake and 290 nmol of O per min·mg for oxygen uptake and thus a scalar H^+/O ratio of 589/290 = 2.03, close to the value 2.0 demanded by Eq. 1. Fig. 4 also shows that the H^+/O flow ratio was close to 2.0 over the entire course of the reaction, demonstrating the reliability of the electrode measurements.

The great power of the membraneless O_2 electrode in monitoring very rapid changes in O_2 is also demonstrated in Fig.

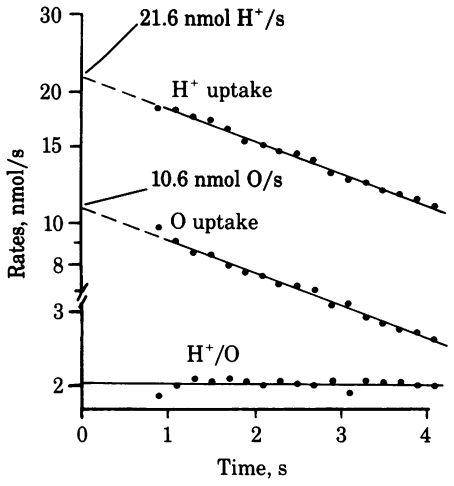

FIG. 4. Semilogarithmic plots of the rates of H^+ uptake (nmol/s) and O_2 uptake (nmol of O per s) vs. time. Multiple points taken from the H^+ and O_2 traces in Fig. 3 at 0.2-s intervals were converted into digital form, and the rates at 0.2-s intervals were computed (nmol/s) and plotted vs. time. The lines were fitted by regression analysis. To calculate the initial H^+/O uptake ratio the rates were extrapolated to zero time, at which ΔpH is zero. Also shown is a plot of the H^+/O uptake ratio vs. time, computed every 0.2 s.

3, which shows in contrast the O_2 trace obtained with a standard Clark electrode in an exactly parallel experiment. Only a very slight upward deflection occurred in the Clark electrode trace, corresponding to less than 2% of the added oxygen.

Vectorial H^+/O Ejection Ratio for the Cytochrome Oxidase Reaction with the Fast O_2 Electrode. The vectorial H^+/O ejection ratio of the cytochrome oxidase reaction was then determined by using the same type of system and the same electrodes and instrumentation used in the validation experiment of Fig. 3, but with FCCP replaced by valinomycin. Under these conditions, vectorial ejection of H^+ rather than scalar H^+ uptake took place (Fig. 5). Successive points taken from the traces at 0.2-s intervals were converted into digital form and the rates of O_2 uptake and H^+ movements were treated as in Fig. 4. Oxygen uptake was again found to be first order up to 70% of its course (Fig. 6), giving a linear semilogarithmic plot vs. time; zero time was set by the known amount of O_2 added. The semilogarithmic plot of the H^+ ejection rate vs. time was also linear (Fig. 6) but for a shorter period because H^+ ejection approached a peak followed by backflow of the ejected H^+ from the medium, which increased as ΔpH across the membrane increased. The earliest reliable points on the H^+ ejection and O_2 uptake traces, after initial mixing and electrode equilibration, gave a directly observed vectorial H^+/O ratio of 3.1 in this experiment. When the semilogarithmic plots were extrapolated to zero time—i.e., to zero H^+ backflow rate—the initial rates (ejection of 1,050 nmol of H^+ per min·mg and uptake of 278 nmol of O per min·mg) gave a vectorial H^+/O flow ratio of 3.78. Fig. 6 also shows the rapid decline in the observed H^+/O ratio with time, owing to H^+ backflow. Many experiments of this type, with some variation of reaction conditions, have repeatedly given extrapolated H^+/O ejection ratios in the range 3.50 to 4.04, closely approaching but never significantly exceeding 4.0.

In the absence of Ca^{2+}, the H^+ and oxygen traces showed essentially no change. When cyanide was added to such systems, both oxygen consumption and H^+ ejection were completely inhibited. H^+ ejection required the presence of a permeant charge-compensating cation, either K^+ plus valinomycin or Ca^{2+}. Observation of maximal H^+/O ejection ratios required an inhibitor of $H^+/H_2PO_4^-$ symport (20) and at least 30 mM K^+ in the reaction medium; much lower K^+ concentrations gave

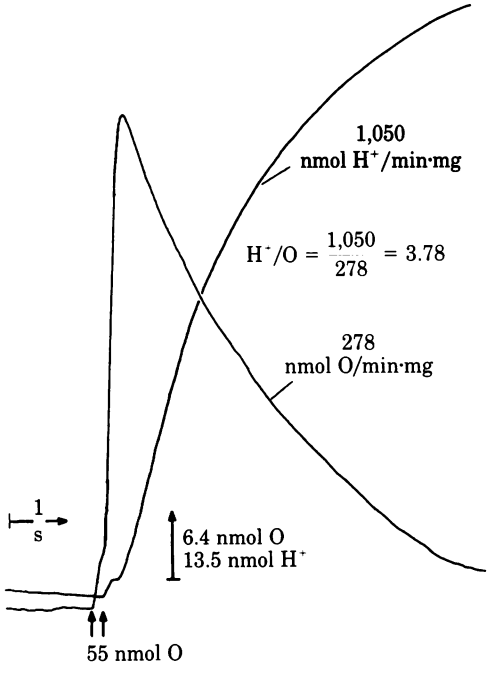

FIG. 5. The vectorial H^+/O ejection ratio (oxygen electrode). The test system was precisely as in Fig. 3 with 4 mg of mitoplast protein and with the FCCP replaced with valinomycin (200 ng/mg). To initiate the reaction, 100 μl of air-saturated medium (17°C) containing oxygen equivalent to 55.0 nmol of O was added to the anaerobic medium. The oxygen uptake and H^+ ejection were monitored as in Fig. 3. The initial rates shown were obtained from the plots in Fig. 6.

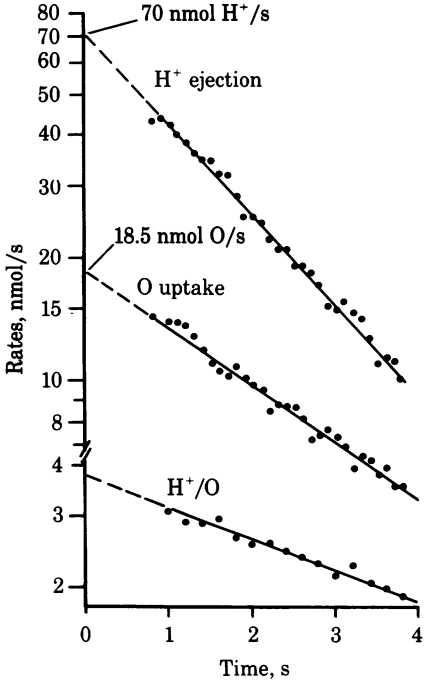

FIG. 6. The H^+/O ejection ratio from semilogarithmic plots of rates of H^+ ejection (nmol/s) and O_2 uptake (nmol of O per s) vs. time. Points taken from the H^+ and O_2 traces in Fig. 5 were processed as described in Fig. 4. Also shown is a semilogarithmic plot, vs. time, of the H^+/O ejection ratio computed every 0.2 s, demonstrating the fast decay of the H^+/O ratio with time.

low H^+/O ratios, no matter how high the valinomycin concentration. Antimycin A, even in very slight excess of that required to inhibit succinate oxidation, lowered the H^+/O ratio substantially through its uncoupling action. When mitoplasts were replaced with intact mitochondria, the rate of oxidation of added C^{2+} and the H^+/O ejection ratio were greatly depressed. The vectorial H^+/O ejection ratios reported here were derived from at least 20 and as many as 70 turnovers of the mitoplast cytochrome oxidase.

DISCUSSION

The data reported here demonstrate, with three different measurement techniques validated against the scalar cytochrome oxidase reaction, that passage of a pair of electrons from C^{2+} to oxygen via cytochrome oxidase of rat liver mitoplasts causes directly observed vectorial ejection of significantly more than 3 H^+ and a maximum close to 4 H^+ when the rates are extrapolated to zero time, at which ΔpH and thus the H^+ backdecay rate both are zero. The new data not only confirm our earlier conclusions (1–3) but also remove the criticism (7, 14) that H^+/O ratios close to 4 are solely the result of underestimation of electron flow with Clark-type O_2 electrodes. They are also compatible with earlier measurements indicating that the average $H^+/2e^-$ ratio for the three energy-conserving sites is close to 4 (19, 21–23). It appears possible that some of the low values for the H^+/O ejection ratio of cytochrome oxidase reported in other studies may have resulted from use of rates obtained from tangents hand-drawn to essentially nonlinear traces, particularly at time points at which the H^+/O ratio had already declined substantially because of the rapid H^+ backflow. Other factors leading to underestimation are excess antimycin A and low K^+ concentrations.

An intrinsic or mechanistic H^+/O ratio approaching 4 for the H^+/O ratio of the cytochrome oxidase reaction, as indicated by the experiments described here, must be taken into account in any self-consistent hypothesis for the structure, mechanism, and subunit orientation of cytochrome oxidase. However, the mechanistic H^+/O ratio may be observable only when electron flow through cytochrome oxidase is initiated at zero load and in all probability does not prevail during oxidative phosphorylation under intracellular conditions. As developed by Stucki (24), the mechanistic H^+/O ratio is most closely approached in the condition of level flow, the transient state in which electron flow is opposed by zero load (i.e., zero electrochemical H^+ gradient across the membrane). The other extreme condition of respiring systems, static head or state 4, prevails when the electrochemical H^+ gradient ($\Delta\mu_{H^+}$) and the phosphorylation potential (ΔGp) are maximal and no net synthesis of ATP occurs but oxygen consumption continues. Oxidative phosphorylation in cells *in situ* ordinarily takes place in steady states poised somewhere between level flow and static head, but generally nearer the latter.

The H^+/O ejection ratios reported here were obtained by the most direct experimental approach to determination of the mechanistic stoichiometry because they involve measurement of the vectorial H^+ produced at conditions very close to level flow. They have been confirmed by independent inhibitor titration experiments capable of setting the upper and lower limits of the H^+/O ratio. Less direct approaches, in which the H^+/O ratio of cytochrome oxidase can be inferred from the stoichiometry of secondary reactions dependent upon vectorial H^+ ejection (namely, net uptake of K^+ or phosphorylation of ADP), have also yielded values of at least 3.0. Full details of these and related studies will be submitted for publication elsewhere.

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