*Discussion Letter*

# The $H^+/O$ ratio of proton translocation linked to the oxidation of succinate by mitochondria

## Reply to a commentary

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Received 16 September 1985

Costa, L.E., Reynafarje, B. and Lehninger, A.L. [(1984) J. Biol. Chem. 259, 4802–4811] have reported 'second-generation' measurements of the $H^+/O$ ratio approaching 8.0 for vectorial $H^+$ translocation coupled to succinate oxidation by rat liver mitochondria. In a *Commentary* in this Journal [Krab, K., Soos, J. and Wikström, M. (1984) FEBS Lett. 178, 187–192] it was concluded that the measurements of Costa et al. significantly overestimated the true $H^+/O$ stoichiometry. It is shown here that the mathematical simulation on which Krab et al. based this claim is faulty and that data reported by Costa et al. had already excluded the criticism advanced by Krab et al. Also reported are new data, obtained under conditions in which the arguments of Krab et al. are irrelevant, which confirm that the $H^+/O$ ratio for succinate oxidation extrapolated to level flow is close to 8.

*Proton* *Stoichiometry* *Electron transport* *Mitochondria* *Succinate*

## 1. INTRODUCTION

In a recent paper Costa et al. [1] concluded that the $H^+/0$ ratio for vectorial $H^+$ translocation during succinate oxidation by rat liver mitochondria is close to 8 at approximate level flow. In their measurements electron flow was initiated by injection of $O_2$ into an anaerobic medium containing mitochondria, succinate, valinomycin, and $K^+$. The rates of $O_2$ uptake and $H^+$ ejection were obtained from $O_2$ and $H^+$ electrodes and recording systems having finite but closely matched response times. In a *Commentary* published in this Journal, Krab et al. [2] have argued from a mathematical simulation that the $O_2$ uptake rates reported by Costa et al. [1] were significantly underestimated, on the grounds that the rapid injection of $O_2$ into the test system produced a large step-increase in $\dot{O}_2$ concentration, from which the $O_2$ electrode had not completely relaxed prior to the measurement of the subsequent $O_2$ uptake rate on succinate, whereas measurement of $H^+$ ejection involved only a gradual rise in $H^+$ concentration.

The general argument advanced by Krab et al. [2] is a valid consideration when 2 measurement systems with finite response times are employed to follow the course of a reaction in which the concentration of one of the reactants is abruptly subjected to a large step-change. However, in this paper we show: (i) that the mathematical model developed by Krab et al. [2] for the simulation of the electrode responses and the kinetics of $H^+$ and $O_2$ changes is not quantitatively correct for the conditions described in [1] and that error estimates

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00145793/85/$3.30 

derived from the model are accordingly in error; (ii) that the criticism of Krab et al. [2] had already been excluded by appropriate control measurements reported by Costa et al. [1]; and (iii) that new experimental methods confirm that the $H^+/O$ ratio extrapolated to zero time is close to 8 for succinate oxidation and close to 4 for the cytochrome oxidase reaction, under conditions in which the criticisms raised by Krab et al. [2] are not relevant.

## 2. EXPERIMENTAL DETAILS

Earlier papers described the preparation of rat liver mitochondria and mitoplasts [1,3], water-jacketed reaction cells [1,4], and fast-responding $O_2$ electrodes [3], which are coated with sintered glass rather than a Teflon membrane. Separate reference electrodes were employed for the $O_2$ and $H^+$ electrodes. In the experiments initiated by photolysis, the reaction vessel was housed in a light-tight Faraday cage. The light beam, furnished by a quartz halogen lamp (Oriel, Stanford, CT, model G333) was focussed through a lens system to provide uniform illumination over the face of the reaction vessel; illumination was initiated by a camera shutter. The electrode signals in the photo-activated experiments were passed through a 12-bit analog/digital converter into an Intel 8080 computer, stored on discs, and processed on a main-frame Digital Equipment system 10 unit. The response times of the electrodes employed in the measurements reported in fig.2 were validated against the scalar cytochrome oxidase reaction [1,3,5].

## 3. RESULTS

### 3.1. *The simulation model of Krab et al. [2]*

The model of Krab et al. [2] assumed as the basis of their computer simulation that a large, instantaneous step-increase of $O_2$ concentration occurred when $O_2$ was injected into the reaction system. However, under the conditions described by Costa et al. [1] manual injection of $O_2$ and mixing of the system are not instantaneous events; they required a significant fraction of the 0.8 s 'dead' time, during which $O_2$ uptake and $H^+$ ejection take place at a high but unknown rate before the system is completely mixed. A properly designed simulation would therefore require knowledge of several factors: (i) the kinetics of injection and mixing of $O_2$, which may be a complex function of the cell geometry, stirring rate, and manual injection time; (ii) the true rate of $O_2$ uptake in the earliest portion of the reaction; (iii) the electrode relaxation constants; and (iv) the precise zero time at which mixing is complete. The system is therefore too complex to allow an accurate simulation, since accurate time constants are not available, particularly for factors (i) and (ii). Therefore the model of Krab et al. [2], which neglected 3 of these essential factors, is grossly oversimplified and cannot predict quantitatively the time required for the $O_2$ electrode to respond to the transient initial rise in $O_2$ and, subsequently, to the decrease in $O_2$ as succinate oxidation proceeds.

Secondly, the mathematical expression of Krab et al. [2] for the simulation of $H^+$ ejection is incorrectly derived. In a model proposed to be quantitative, $H^+$ back-flow cannot be accounted for simply by adjusting the rate constant for $H^+$ ejection in the manner given by eqns 7 and 8 of Krab et al. [2], since $H^+$ back-flow increases as $H^+$ ejection decreases. Quantitative treatment of the recorded rate of $H^+$ ejection in the manner proposed requires 3 exponential terms (i.e. for electrode response, $H^+$ ejection, and $H^+$ back-flow) rather than the 2 exponential terms used by Krab et al. [2]. Therefore their subsequent derivation of eqn 13 for the error factor $F$ in the determination of the $H^+/O$ ratio is incorrect, as are the conclusions drawn from its application.

Third, the expression for the error factor $B$ for the Guggenheim method of rate approximation, given by eqn 11 of Krab et al. [2], which describes the effect of the time increments used by Costa et al. [1], is also incorrect, not only because of the incorrect modeling of $H^+$ back-flow but particularly because of its mathematical form. Since error factor $B$ is obtained from increments of time rather than increments of rate, it is correctly given by a McLaurin expansion, which reduces to the equation

$$B = \frac{1 + 1/24\ (k_H \Delta t)^2}{1 + 1/24\ (k_O \Delta t)^2}$$

Using the values assumed by Krab et al. [2] for $k_H$, $k_O$, and $\Delta t = 0.2$ s, the error factor $B$ is correctly evaluated as 1.00005, i.e. there was no significant

error in the estimation of the $H^+/O$ ratio from Guggenheim plots by Costa et al. [1].

### 3.2. *Validation of the $H^+$ and $O_2$ recording systems by Costa et al. [1]*

In their paper, Costa et al. [1] had in any case already excluded the type of argument advanced by Krab et al. [2]. First, they employed closely matched electrodes; the 90% response time of the $O_2$ electrode was 0.8 s and that of the glass electrode 0.9 s. Both electrodes exhibit precisely first-order response characteristics (Hendler, R.W., Shrager, R.I., Setty, O.H., Reynafarje, B. and Lehninger, A.L., to be published). Secondly, Costa et al. [1] demonstrated the reliability of their electrode recording systems by measurements on the known $H^+/O$ stoichiometry of the *scalar* cytochrome oxidase reaction in rat liver mitoplasts in the presence of ferrocytochrome *c* and a protonophore, which proceeds according to the equation

$$2 \text{ cyt. } c^{2+} + 2 H^+ + \tfrac{1}{2}O_2 \longrightarrow 2 \text{ cyt. } c^{3+} + H_2O \quad (1)$$

The reaction was initiated by initiated by injection of $O_2$, exactly as in their vectorial $H^+/O$ ratio measurements. Costa et al. [1] found their electrode recording systems to yield scalar $H^+/O$ uptake ratios very close to the theoretical value of 2.0 required by reaction 1 from the end of the dead time (~0.8 s after injection) to at least 90% of the total reaction course (fig.1 of Costa et al. [1]); such close agreement was found at rates of $O_2$ uptake up to and exceeding 25 nmol O/s. Similar tests demonstrating the reliability of the electrode systems had been reported earlier by Reynafarje et al. [3] and again more recently [5], in the latter case using the scalar reaction for NADH oxidation by uncoupled submitochondrial preparations:

$$NADH + H^+ + \tfrac{1}{2}O_2 \longrightarrow NAD^+ + H_2O$$

These tests, inexplicably unmentioned by Krab et al. [2], demonstrated that by the end of the dead time of ~0.8 s the $O_2$ electrode system had already relaxed sufficiently from the initial injection of $O_2$ so that the 2 electrodes were thereafter competent to measure the $H^+/O$ ratio accurately.

### 3.3. *Vectorial $H^+/O$ ratios determined in systems not involving rapid initial injection of $O_2$: photoactivation of carbon monoxide-inhibited succinate oxidation*

Two types of vectorial $H^+/O$ flow ratio measurements are now described which totally avoid an initial rapid injection of $O_2$ and thus make irrelevant the criticism advanced by Krab et al. [2]. In the first the oxidation of succinate was initiated in the basic test system of Costa et al. [1]. However, instead of initiating electron flow by injection of $O_2$, it was accomplished, virtually instantaneously, by photolysis of the carbon monoxide-inhibited cytochrome *c* oxidase of the mitochondria, after preincubation of the complete system in the dark under an atmosphere of 100% CO in the presence of succinate and 10 $\mu$M $O_2$. Under these conditions no step-change in $[O_2]$ occurs when the system is illuminated. The electrode signals were directly accessed into a computer at intervals of 10 ms using about 1000 data points/electrode per experiment. Electronic and mechanical noise were drastically reduced by a combination of techniques, including electronic filtration. The experimentally determined relaxation constants ($\tau$) were close to 0.2 s for both electrodes ($\tau$, time required to reduce the error to $1/e$) . After photoactivation of electron flow and $H^+$ ejection the rates of both $O_2$ uptake and $H^+$ ejection by rat liver mitochondria could be fitted to monotonic exponentials over a period of 0.8 to at least 3 s, from which the zero time rates could be extrapolated. The data from a representative experiment, plotted in the form used by Costa et al. [1], are shown in fig.1. From a total of 31 experiments $H^+/O$ flow ratios at approximate level flow obtained in this way were 8.0 ± 0.4, in good agreement with the values of 7.3–7.9 reported by Costa et al. [1]. Moreover, the virtual elimination of the stirring noise, to which all $O_2$ electrodes are subject, yielded plots with very little scatter, thus removing another criticism advanced by Krab et al. [2].

### 3.4. *Initiation of oxidation by injection of the electron donor rather than $O_2$*

A second way of avoiding abrupt changes in $O_2$ concentrations is to initiate electron flow by injecting the electron donor into the already aerobic system containing an excess of $O_2$, conditions under which no abrupt change in $[O_2]$ occurs and

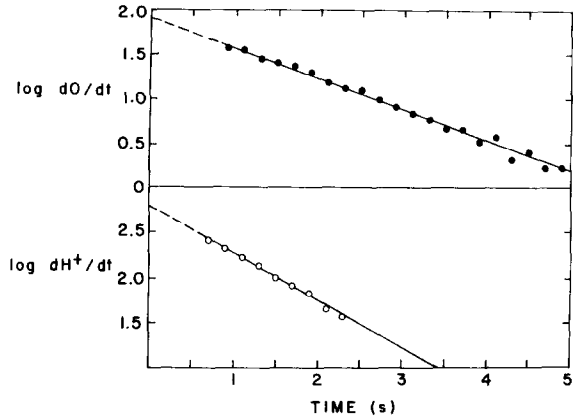


Fig.1. $H^+$ and $O_2$ electrode data obtained when succinate oxidation was initiated by photoactivation of CO-inhibited electron flow through cytochrome *c* oxidase. The basic medium of 50 mM KCl, 200 mM sucrose, 1.5 mM $K^+$-Hepes, pH 7.05 (6.0 ml, $t$ = 25°C) in a glass reaction vessel was made nearly anaerobic with a stream of $N_2$ and 100% CO then passed over the surface at a rate of 80 ml/min. Rotenone (2 $\mu$M) and succinate (5.0 mM) were added in microliter volumes followed by rat liver mitochondria (18 mg protein) in 0.3 M sucrose. $O_2$ admitted during the additions was depleted by illumination of the suspension until the $O_2$ trace was essentially zero; 18 $\mu$g oligomycin, 90 $\mu$g *N*-ethylmaleimide and 2.2 $\mu$g valinomycin were then added. Illumination was stopped and the suspension incubated in the dark for 20 min under CO. A known amount of $O_2$ (45.0 nmol O) in air-saturated medium was added in the dark; this addition also served for calibration of the $O_2$ electrode. After further dark incubation (30 s), which established the zero time baselines, the shutter was opened to initiate electron flow. The pH electrode was calibrated at the end of the experiment with 400 nmol HCl. The signals for both electrodes were accessed directly into the computer at 10-ms intervals. Data from 0.8 to 5 s for the oxygen electrode and from 0.8 to 3 s for the pH electrode were fitted to the rate expression $S = Ae^{-kt} + A_\infty$ for evaluation of the parameters $A$, $k$, and $A_\infty$. The raw data for the rates $(X)$ as well as the continuous plots obtained from the best-fit parameters are shown in semi-log form for the $O_2$ electrode (upper panel) and the $H^+$ electrode (lower panel). The extrapolated $H^+/O$ ratio at true zero time was 7.83.

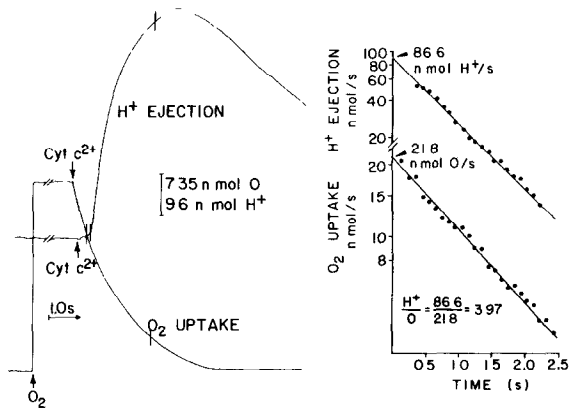


Fig.2. The $H^+/O$ ratio for the cytochrome oxidase reaction initiated by ferrocytochrome *c*. The reaction system (1.50 ml, $t$ = 10°C) consisted of 50 mM KCl, 200 mM sucrose, 3.0 mM $K^+$-Hepes pH 7.10, antimycin A (0.1 nmol/mg), *N*-ethylmaleimide (40 nmol/mg), valinomycin (300 ng/mg), about 10 $\mu$M $O_2$, and rat liver mitoplasts (2.5 mg protein). After stabilization of the electrode traces 180 nmol cyt. $c^{2+}$ was injected to initiate electron flow and the $O_2$ uptake and $H^+$ ejection recorded as shown in the reaction traces. Points were taken at 0.1-s intervals from the unsmoothed traces between the vertical bars, digitized, and shown in the form of Guggenheim plots (right), fitted by a weighted regression analysis. The time at which mixing was complete in this experiment (i.e. zero time) was approximated to be 0.1 s after start of the injection of cyt. $c^{2+}$. This approximation was made possible because the $O_2$ electrode employed had a 90% response time of $<0.1$ s, as can be seen from the rapid response of the $O_2$ trace to the injection of $O_2$. However, since the $H^+$ electrode had a much longer 90% response time ($\sim 0.6$ s), only the data taken from comparable points of the traces beginning at 0.5 s were used to extrapolate the initial $H^+/O$ ratio, which was 3.97 at 0.1 s.

the criticism of Krab et al. [2] does not pertain. Fig.2 shows the data obtained from a typical experiment in which electron flow through cytochrome oxidase was initiated by injection of cyt. $c^{2+}$ into an already aerobic suspension of rat liver mitoplasts under the conditions described in [3], in order to determine the $H^+/O$ ratio for the cytochrome oxidase reaction, for which this [3,6] and another laboratory [7,8] have found $H^+/O$ ratios approaching 4, but for which Krab et al. [2] support a value of 2. This type of experiment directly engages the real issue at hand: the $H^+/O$ ratio for the cytochrome oxidase reaction. Since there is general agreement that the $H^+/2\ e^-$ ratio of electron flow from succinate to cyt. $c^{3+}$ or ferricyanide is 4 (for references see [9,10]), the difference between $H^+/O$ ratios of 6 and 8 for succinate oxidation reflects the difference between $H^+/O$ ratios of 2 and 4 for the cytochrome oxidase reaction. The

Guggenheim plots in fig.2 show that both $O_2$ uptake and $H^+$ ejection rates obtained from the electrode traces of such an experiment can be fitted to single exponentials from the end of the dead time (~0.4 s) to over 85% of the maximum observed $H^+$ ejection. Extrapolation of the plots to the approximated zero time (complete mixing) yielded and $H^+/O$ ratio of 3.90. Thus, using either the photolysis measurements described in fig.1 or the manual injection procedure used in fig.2, the extrapolated $H^+/O$ ratio of close to 8 for succinate oxidation is supported under conditions in which the considerations developed by Krab et al. [2] are not applicable.

It is emphasized here, although this obvious point was not explicitly developed by Costa et al. [1], that when the reaction is started by mixing either $O_2$ or electron donor with the rest of the system, as in the experiment in fig.5 of [1] and fig.2 of this paper, and in earlier reports [1,3,5], the true zero time of the measured reaction, i.e. the time at which the electrodes first 'see' the fully mixed system, is not the time when the manual injection of the last reagent is begun (arrows), since both $O_2$ uptake and $H^+$ ejection take place at high but unknown and unpredictable rates during the finite time required for mixing. For the same reason the exact concentration of $O_2$ (or cyt. $c^{2+}$) after mixing is just complete is not precisely known but is obviously less than the amount of $O_2$ or cyt. $c^{2+}$ that was injected. The time required for manual injection and complete mixing varies from experiment to experiment, up to 0.3 s after injection of $O_2$ is begun. While plots of the extent of $O_2$ uptake vs time sometimes yield intercepts of initial $O_2$ concentrations close to the amount actually added, more usually they are significantly less, as can be expected from the considerations just developed. As can be seen from the scale of plot A of fig.5 of Costa et al. [1], the $O_2$ concentration obtained by extrapolation to the time when injection was begun was incorrectly labeled 48.2 nmol O; as can be seen the correct value is 44.5 nmol O. Because Krab et al. [2] were led to assume that the zero time $O_2$ concentration was that of the added $O_2$, an incorrectly high value of the rate constant for $O_2$ uptake was obtained. Since neither the $O_2$ concentration after mixing nor the final concentration of ejected $H^+$ is precisely known or predictable in such experiments, use of Guggenheim plots (log $\Delta O/\Delta t$ and log $\Delta H^+/\Delta t$ vs time) by Costa et al. [1] is an appropriate procedure, contrary to Krab et al. [2]. The inherent error in fixing zero time and the initial $O_2$ concentration, characteristic of the type of measurement described by Costa et al. [1] and that in fig.2, tends to underestimate the extrapolated level flow $H^+/O$ ratio slightly. This uncertainty is eliminated by the photolysis technique as described in fig.1 of this communication.

## 4. CONCLUSIONS

On the basis of: (i) the inappropriate model and inexact simulation developed by Krab et al. [2]; (ii) the demonstrated capacity of the $O_2$ and $H^+$ electrode systems used by Costa et al. [1] to record $H^+$ and $O_2$ changes reliably [1,3,4]; and (iii) the new measurements in figs 1 and 2, we reject the conclusion of Krab et al. [2] that $H^+/O$ ratios approaching 8 for succinate oxidation reported by Costa et al. [1] are the result of gross overestimation. The new measurement techniques briefly described here represent additional experimental approaches to the determination of the $H^+/O$ ratio of mitochondrial electron transport at level flow, which support earlier measurements of the $H^+/O$ ratios for succinate and cyt. *c* oxidation from the Baltimore laboratory [3,6,11–13], as well as independent data on $H^+$ stoichiometry derived from other types of kinetic and thermodynamic measurements employed in other laboratories [7,8,14–17].

The methods described here and in [1,3,5] have the advantage of indicating the magnitude and rate of the energy leaks, but the disadvantage of requiring extrapolation to zero leak to obtain the true $H^+/O$ ratio. A theoretical rationale and supporting data for a generally applicable method for determination of stoichiometric ratios of energy-coupled transmembrane transport processes, which does not require knowledge of the nature or rate of energy leaks, will be presented for publication elsewhere (Beavis, A and Lehninger, A.L.).

The mechanistic $H^+/O$ ratio, i.e. the maximum stoichiometry for which a given electron transport system provides a coupling mechanism, is in principle never directly observable from flow ratios alone or force ratios alone [17,18]; in both cases some form of correction or extrapolation to account for energy leaks and/or incomplete coupling is required. Krab et al. [2] reported that their

$H^+/O$ ratio of 6 for succinate oxidation is in agreement with the observations of others. However, in neither their studies nor the reports they cited was the $H^+/O$ ratio determined under conditions that satisfactorily allow for energy leaks, other forms of incomplete coupling, or pump slippage [17]. The classical $O_2$ pulse method, which does not actually measure $O_2$ uptake, necessarily underestimates the mechanistic $H^+/O$ ratio, because the amount of $H^+$ ejected per unit of $O_2$ added is obtained by back-extrapolation of the observed $H^+$ ejection to a time point at which significant $H^+$ ejection and $O_2$ uptake had already occurred, among other reasons [19,20]. Krab et al. [2] also failed to mention that other laboratories, using a variety of kinetic and thermodynamic approaches capable of accounting for energy leaks, have given quantitative support to a value of 8 for the $H^+/O$ ratio of succinate oxidation [7,8,14–17].

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