THE H^+/O STOICHEIOMETRY OF MITOCHONDRIAL RESPIRATION

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1. Introduction

Mitochondrial respiration is compulsorily linked to proton ejection [1,2]. The mechanism by which transmembrane $\Delta\tilde{\mu}H^+$ is generated is, however, unknown [3]. Essential for exploring this issue is knowledge of the quantitative relationship between proton translocation and electron transport.

Numerous determinations in mitochondria and bacteria produced a stoicheiometry of 2 H^+ translocated for 2 e^- traversing an effective protonmotive redox loop, or energy conserving site of respiratory chain (reviewed [1,2,4]). Recently, however, the $H^+/2\ e^-$ and H^+/ATP stoicheiometries have been the subject of much controversy [2,5–9]. Thus contrasting results have been reported, which indicate that in mitochondria the H^+/O quotient for succinate or quinol respiration (sites 2 + 3) is either 4 [1,7,10–14], or 6 [6,8,15], or 8 [5,9,16,17].

We present here accurate spectrophotometric determination of the rate of respiration with hemoglobin and potentiometric determination of the acidification of the extramitochondrial space, which unequivocally show that the H^+/O quotient for oxidation of succinate in mitochondria is, at neutral pH, 4.

2. Methods

Rat-liver mitochondria [14], were incubated in a standard reaction mixture containing: 130 mM LiCl, 1 mM KCl, 3 mM Hepes, valinomycin (0.1 μg/mg protein) and *N*-ethylmaleimide (30 nmol/mg protein). Samples of the same mitochondrial suspension (pH 7.2 ± 0.005) were transferred to thermostatically controlled (25 ± 0.01°C) glass cell, equipped with Clark oxygen electrode (4004 YSI, Yellow Spring, OH), coated with a standard membrane (YSI 5775) or a high sensitivity membrane (YSI 5776) [18], and combination glass electrode (10405328, Ingold, AG, Zürich), and a spectrophotometric cuvette (1 cm lightpath). The accuracy of the pH-recording device used was 0.001 pH unit, the response time 0.4 s/pH unit [19]. When desired the concentration of dissolved O_2 was lowered by blowing argon onto the surface of the vigorously stirred suspension.

The cells were then sealed with glass plugs with 3 cm long, thin channels, filled with mitochondrial suspension, for insertion of electrodes and microsyringe needles.

Spectrophotometric assay of respiration with hemoglobin was done by the method of [20] as in [18]. Concentration of human hemoglobin, prepared as in [21], was estimated, on the heme basis, with an ϵ_{mM} at 577 nm of 15.4 [20]. Deoxygenation of HbO_2 was monitored as decrease in $A_{577-568}$ ($\Delta\epsilon_{mM} = 4.8$) with a dual wavelength spectrophotometer [18]. The rate of oxygen consumption in natoms/min was obtained by multiplying the rate of HbO_2 deoxygenation (in nmol heme) by 2 and a correction factor (f). This was calculated polarographically and spectrophotometrically, and for rat-liver mitochondria amounted, with both methods, to 2.46 at 25 μM hemoglobin [cf. [18,20]).

3. Results

Fig.1 shows a typical course of respiration and H^+ ejection initiated by succinate pulses of mitochondria suspended in the LiCl medium, equilibrated with

Abbreviations: HbO_2, oxyhemoglobin; Hb, deoxyhemoglobin

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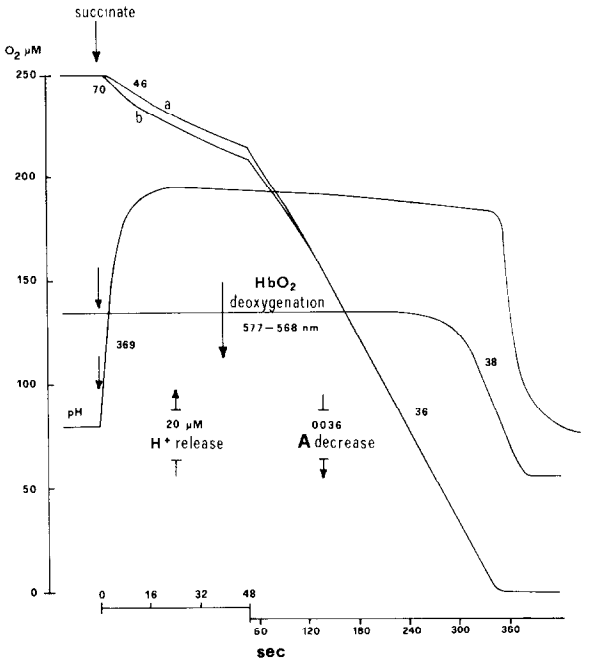

Fig.1. Respiration and proton release elicited by succinate addition to air-saturated rat-liver mitochondria. Mitochondria (2.5 mg protein/ml) were suspended in the standard reaction mixture supplemented with rotenone (0.5 μg/mg protein). The suspension, transferred to the measuring cells, was pre-incubated for 5 min, then 1 mM succinate was rapidly injected. (a) Standard membrane; (b) high sensitivity membrane. The suspension in the spectrophotometric cell was supplemented with 25 μM HbO_2. Controls showed that HbO_2 had no effect per se on the initial rates of oxygen consumption, measured polarographically, and proton release. pH-changes were converted in ng ions H^+ by double titration with standard solutions of HCl and KOH. Oxygen concentration was calibrated as in [18]. It can be noted that addition of succinate to the HbO_2 containing suspension did not cause any $\Delta A_{577-568}$. Furthermore in the absence of HbO_2 no $\Delta A_{577-568}$ was observed when mitochondria become anaerobic. The mitochondrial suspension containing 25 μM HbO_2 took more time to reach anaerobiosis than the sample without HbO_2, because of the extra-oxygen donated by HbO_2. The numbers on the traces are rates expressed as ng atoms oxygen consumed and ng ions H^+ translocated . min^{-1}. mg $protein^{-1}$.

atmospheric oxygen and supplemented with rotenone, NEM and valinomycin (plus K^+) (cf. [5,16]). Succinate caused a rapid acidification which reached a steady state in ~30 s. With anaerobiosis pH returned to the initial level. The succinate-induced H^+ release, was abolished by antimycin or nigericin (not shown).

The O_2 electrode coated with the standard membrane responded to respiration elicited by succinate with a lag of 3 s. Respiration exhibited an initial, transitory rapid phase. With the electrode coated with the high sensitivity membrane the lag was 1.5 s. Respiration exhibited the same pattern observed with the standard membrane. However it can be noted that whilst the rates of O_2 consumption measured with the two membranes in the late respiratory phase were equal, the initial respiratory rate obtained with the high sensitivity membrane was 50% higher than that measured with the standard membrane. Considering its intrinsic response time [8,18], the O_2 electrode, coated with either membrane, appears to be inadequate to measure accurately the initial and transient rapid rate of respiration. This is shown to be the case by comparison of polarographic and spectrophotometric measurements of respiratory rates.

Deoxygenation of 25 μM HbO_2, added to the mitochondrial suspension respiring with succinate, started when dissolved O_2 was reduced to ~90 μM and proceeded with a linear slope from 25–80% deoxygenation, corresponding to 50–10 μM effective O_2 concentration (dissolved O_2 plus O_2 contributed by HbO_2). The respiratory rate obtained from the slope of the linear tract of the HbO_2 deoxygenation curve (see section 2 and [18,20]) was equal to that measured polarographically in the terminal respiratory phase.

Estimation of the correct H^+/O quotient requires exact spectrophotometric determination of the initial respiratory rate as it ensues immediately upon succinate addition. This was obtained by pre-lowering with argon the concentration of dissolved O_2 in the incubation mixture until HbO_2 was 25–50% deoxygenated (corresponding to effective O_2 concentrations of 50–27 μM).

Fig.2A shows that addition of succinate to a mitochondrial suspension, where O_2 was reduced to 27 μM, elicited a respiratory activity (as measured polarographically) and H^+ translocation similar to that observed in the mitochondrial suspension equilibrated with atmospheric oxygen. Under these conditions the respiratory burst elicited by succinate resulted in immediate deoxygenation of hemoglobin, as shown by the abrupt $A_{577-568}$ decrease. The respiratory rate, calculated from that of HbO_2 deoxygenation, exhibited an initial rapid phase after which declined markedly in a few seconds.

Table 1 gives a statistical analysis of experiments like those illustrated in fig.1,2. It is shown that:

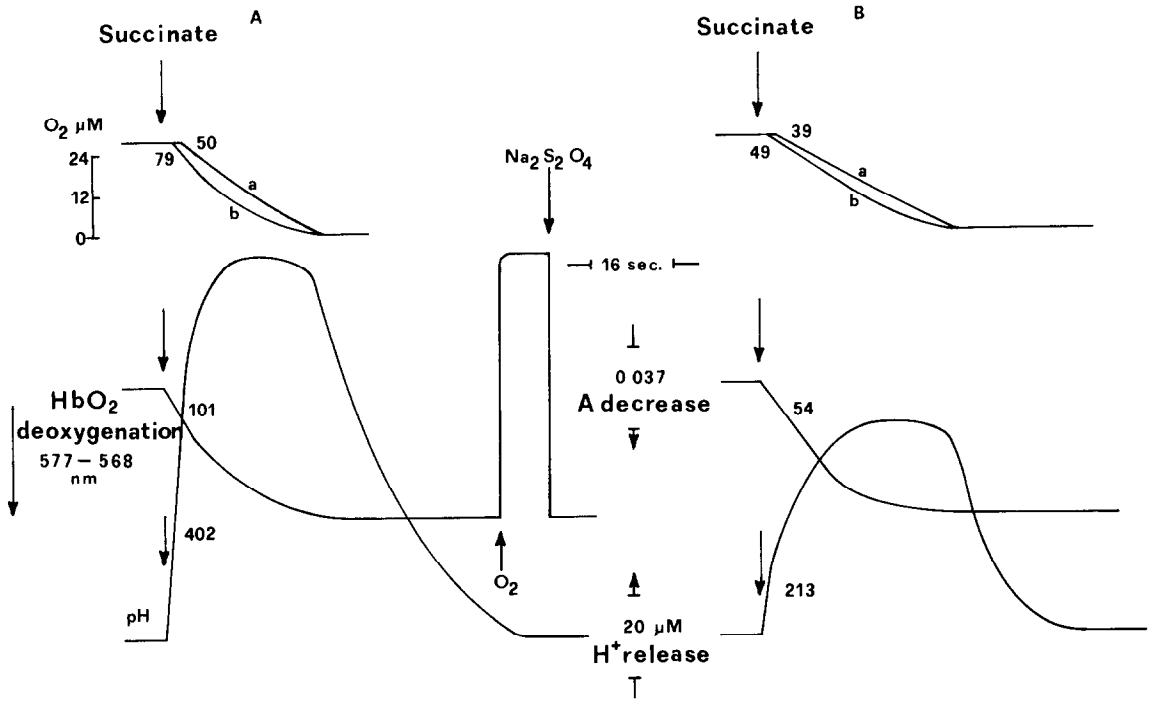

Fig.2. Respiration and proton release elicited by succinate addition to rat-liver mitochondria at reduced O_2 concentration.

(A) Mitochondria were suspended in the mixture of fig.1. Argon was blown onto the surface of the mitochondrial suspension for 4 min, until dissolved O_2 in the polarograph cell sample was reduced to 27 μM and the 25 μM HbO_2, added spectrophotometric cell sample, was 50% deoxygenated. After 1 min further preincubation for temperature equilibration at 25°C, 1 mM succinate was rapidly injected.

(B) All the conditions were the same as in (A) except that oligomycin (1 μg/mg protein) was included in the reaction mixture from the beginning of preincubation and rotenone was added only 1 min before succinate addition. (a) Standard membrane; (b) high sensitivity membrane. After anaerobiosis, hemoglobin was fully reoxygenated by blowing pure oxygen onto the surface of the mitochondrial suspension. The $\Delta A_{577-568}$ caused by oxygenation was equal to that obtained by subsequent deoxygenation with $Na_2S_2O_4$. Controls showed that in the absence of hemoglobin the above treatment of the mitochondrial suspension did not cause any ΔA. It can be also noted from the polarographic and the $A_{577-568}$ trace, recorded before succinate addition, that there was no oxygen diffusion in the suspension after dissolved O_2 had been lowered to <30 μM by argon. The numbers on the traces are rates expressed as ng atoms oxygen consumed and ng ions H^+ translocated . min^{-1}. mg $protein^{-1}$.

(i) Lowering dissolved oxygen from 250 to 25–30 μM had no effect on the initial rates of respiration and H^+ translocation;

(ii) The initial rate of succinate respiration measured spectrophotometrically was twice as high as that measured polarographically with the standard membrane and 30% higher than that measured with the high sensitivity membrane;

(iii) The H^+/O quotient for succinate respiration was 8 when obtained from polarographic measurement of respiration with the standard membrane (cf. [5,16]), 5.2 with the high sensitivity membrane and 4 with spectrophotometric measurement of oxygen consumption.

The H^+/O quotient, obtained either from polarographic or spectrophotometric measurement of oxygen consumption, did not change when mitochondrial protein was varied from 1.0–3.5 mg/ml (fig.3).

In agreement with [10,22], when mitochondria were preincubated aerobically with valinomycin and oligomycin, in the absence of rotenone, the H^+/O quotient for succinate respiration, calculated with polarographic assay of the respiratory rate, was much lower than 8:5.6 with the standard membrane and 4.3 with the

Table 1
Statistical analysis of the initial rates of respiration and proton release and of H^+/O quotients for succinate oxidation in rat-liver mitochondria

Expt.	H^+ release (ng ions . min^{-1} . mg protein^{-1})	Oxygen consumption (ng atoms . min^{-1} . mg protein^{-1})			$\leftarrow H^+/O$		
		(a) Polarographic (standard membrane)	(b) Polarographic (high-sensitivity membrane)	(c) Spectrophotometric (25 μM hemoglobin)	(a)	(b)	(c)
(i)	(12) 397 ± 22	46.5 ± 3.0	77.4 ± 8.8		8.07 ± 0.34	5.49 ± 0.18	
(ii)	(20) 424 ± 18	53.2 ± 2.7	82.0 ± 7.0	106.0 ± 4.5	7.92 ± 0.11	5.21 ± 0.16	4.04 ± 0.05
(iii)	(9) 218 ± 4	38.3 ± 1.1	50.2 ± 1.3	54.2 ± 1.9	5.65 ± 0.12	4.29 ± 0.16	4.02 ± 0.17

The values are the means ± SEM for no. expt indicated in brackets. The values for the H^+/O ratio are means ± SEM of the internal H^+/O ratio measured in different experiments. Experimental conditions: (i) see fig.1; (ii) see fig.2A; (iii) see fig.2B. The means in (ii) and (iii) are from experiments in which, at the time of succinate addition, the concentration of dissolved O_2 was 27 (±2) μM and hemoglobin was deoxygenated by 50% (±5%). The concentration of hemoglobin was in all the experiments 25 μM ($f = 2.46$). Experiment with 35 μM HbO_2 ($f = 2.1$) produced exactly similar results. For details see fig.1,2 and section 2

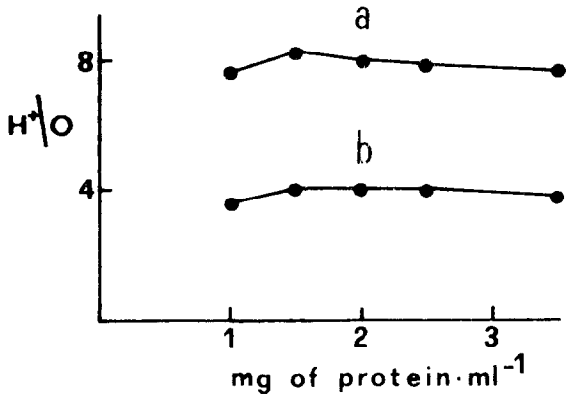

Fig.3. H^+/O ratios for succinate respiration at different concentrations of rat-liver mitochondria. The H^+/O ratios were obtained from (a) polarographic measurement (standard O_2-electrode membrane) and (b) spectrophotometric assay of initial respiratory rates at 27 μM dissolved O_2 and 50% deoxygenation of 25 μM hemoglobin. For experimental conditions see fig.1,2.

high sensitivity membrane. The H^+/O obtained from spectrophotometric assay of respiration remained, on the contrary, equal to 4 under these conditions (fig.2B, table 1). This is due to the fact (table 1) that whereas the initial respiratory rate, measured spectrophotometrically, and the rate of proton release were both depressed by 50%, the respiratory rates measured polarographically exhibited a 37% (high sensitivity membrane) and 28% (standard membrane) depression as compared to those recorded under standard conditions (aerobic preincubation in the presence of rotenone).

These observations confirm that the apparent high H^+/O quotient obtained with polarographic assay of respiration, in the conditions of [5,16] (preincubation with rotenone), is due to failure of the oxygen electrode to record accurately an initial and transient rapid phase of respiration. Preincubation in the absence of rotenone abolishes this phase, succinate respiration proceeds at a constant rate for a longer interval and the rate measured by the electrode better approaches the actual value.

4. Discussion

The results presented show that the H^+/O quotient for succinate respiration in mitochondria is 4. This estimate is based on accurate spectrophotometric determination of the initial rate of respiration and potentiometric determination of the initial rate of H^+ ejection, elicited by succinate addition to aerobic mitochondria under conditions chosen to minimize back-diffusion of H^+ through the membrane (cf. [5,16]).

It is demonstrated that previous estimates, with this steady state rate method, of H^+/O quotients higher than 4 are invalid since resulting from underestimation of the initial respiratory rate [5,16]. The respiratory rate elicited by succinate in mitochondria preincubated with rotenone [5,16] is characterized by an initial, transient rapid phase, which declines in a few seconds (fig.1,2A). This can be accurately determined by the hemoglobin method, which has an intrinsic response $t_{1/2}$ of 0.1 s [18], but is underestimated by the polarographic method (see table 1), which has a lag of 3 s and 1.5 s and response $t_{1/2}$ of 3 s and 1–0.5 s with standard and fast membrane, respectively [8,15,18,23].

The rapid phase of succinate respiration is probably sustained by rapid succinate uptake in exchange with P_i and anionic substrates, accumulated in the matrix during preincubation with NEM and rotenone. It disappears when mitochondria are preincubated aerobically in the absence of rotenone and presence of oligomycin, conditions which allow aerobic depletion of endogenous substrates and esterification of P_i through substrate level phosphorylation (this process is oligomycin-insensitive, differing from ATPase activity).

NEM has been found to enhance the $\leftarrow H^+/2\ e^-$ (per site) quotient, measured with the respiratory pulse method, from 2–3 [5,6]. Hence it was concluded that $\leftarrow H^+/2\ e^-$ (per site) quotient of 2, previously obtained with this method (reviewed [1,2,4]), was underestimated [5,6,8,24]. Mitchell at al. [7,23,25,26] have rejected this claim and proposed that the enhancement of the $\leftarrow H^+/2\ e^-$ quotient caused by NEM is, in these experiments, due to activation of H^+ translocating oxidation of endogenous NAD(P)H, rather than to prevention of H^+ backflow [5,6,8,24].

It is agreed that the $\leftarrow H^+/2\ e^-$ quotient for electron flow from succinate, or quinol, to cytochrome c^{3+} is, at neutrality, 4 [7–11,17,24]. This, together with the demonstration of an H^+/O quotient of 4 for succinate oxidation (or quinol oxidation [10,11,27]), leads to the inescapable conclusion that electron flow at the third site from cytochrome c^{2+} to oxygen does not cause H^+ ejection from mitochondria.

Thus these results are consistent with and reinforce independent observations [11–14,26,28] providing evidence that antimycin-insensitive H^+ release from mitochondria, elicited by aerobic oxidation of reductants of cytochrome *c* oxidase [8,9,15,17], results from redox reactions of respiratory carriers on the substrate side of cytochrome *c*, or from oxidation of the same reductants, but not from redox H^+ pumping by the oxidase as proposed in [8].

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