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The cytochrome chain of mitochondria exhibits variable H^+/e^- stoichiometry

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A study is presented of the $\leftarrow H^+/e^-$ stoichiometry for H^+ pumping by the cytochrome chain in isolated rat liver mitochondria under level-flow and steady-state conditions. It is shown that the $\leftarrow H^+/e^-$ stoichiometry for the cytochrome chain varies under the influence of the flow rate and transmembrane $\Delta\mu H^+$. The rate-dependence is shown to be associated with cytochrome *c* oxidase, whose $\leftarrow H^+/e^-$ ratio varies from 0 to 1, whilst the $\leftarrow H^+/e^-$ ratio for the span covered by cytochrome *c* reductase is invariably 2.

Mitochondria; Cytochrome chain; H^+ pumping; Cytochrome *c* oxidase; Cytochrome *c* reductase

1. INTRODUCTION

Electron flow down the cytochrome chain of mitochondria is compulsorily linked to vectorial H^+ translocation from the inner to the outer aqueous space [1,2]. The mechanism by which $\Delta\mu H^+$ is generated by mitochondrial respiration and utilized to make ATP [3] is, however, not yet completely understood. Critical in this respect is knowledge of the H^+/e^- and H^+/ATP stoichiometries (for review see [4,5]).

The $\leftarrow H^+/e^-$ stoichiometry for electron flow in the cytochrome *c* reductase (span from ubiquinol to cytochrome *c*) is reported to be 2 [4,5]. The $\leftarrow H^+/e^-$ stoichiometry for H^+ ejection by cytochrome oxidase (span from cytochrome *c* to oxygen) is, on the contrary, still debated [6]. Some authors maintain that the $\leftarrow H^+/e^-$ ratio is invariably 1 [5,7,8]. Others have reported $\leftarrow H^+/e^-$ ratios for H^+ ejection by the oxidase lower [4,6] or higher [9,10] than 1. In this paper an analysis is presented of the phenomenological $\leftarrow H^+/e^-$ stoichiometry for respiratory H^+ pumping in rat-liver mitochondria under level flow and steady-state conditions [11]. For level flow, that is, conditions of negligible transmembrane $\Delta\mu H^+$ (see [11]), spectrophotometric measurements of the intitial rates of e^- flow [12] and H^+ translocation elicited by substrate addition were used. The $\leftarrow H^+/e^-$ ratios at the steady-state were measured with a method our group had previously introduced [13].

The results show that the $\leftarrow H^+/e^-$ stoichiometry for succinate respiration varies from around 2 to 3 under the influence of the rate of electron flow and $\Delta\mu H^+$. The rate-dependence is shown to be specifically associated with H^+ pumping the cytochrome oxidase. These observations seem to solve the controversy so far registered for the $\leftarrow H^+/e^-$ stoichiometry in the oxidase.

Abbreviations: TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine; CCP, carbonylcyanide-*m*-chlorophenylhydrazone; NEM, *N*-ethylmaleimide; HEPES, *N*-[2-hydroxyethyl]piperazine-*N′*-[2-ethanesulfonic acid]; Hb, deoxyhemoglobin; HbO_2, oxyhemoglobin; $\leftarrow H^+/e^-$, number of H^+ equivalents released from mitochondria per equivalent e^- transfer.

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2. MATERIALS AND METHODS

Rat-liver mitochondria were isolated as in [14]. H^+ translocation was measured either potentiometrically [15] or by dual-wavelength spectrophotometry at 558–593 nm with the pH indicator phenol red. Oxygen uptake was measured either electrometrically by fast responding O_2 electrode or spectrophotometrically by human hemoglobin as in [12] (see also [16]). Deoxygenation of HbO_2 was monitored by dual wavelength spectrophotometry at 577–568 nm ($\Delta\epsilon$ (mM) for hemoglobin deoxygenation was 6.3). The rate of electron transfer was obtained by multiplying the rate of HbO_2 deoxygenation (in nmol heme) by 4 and a correction factor (*f*). This was calculated polarographically and spectrophotometrically as in [16] (see also legend to Fig. 1). Measurements were carried out in thermostatically controlled glass cells equipped with O_2 electrodes (4004 YSI, Yellow Spring, OH), coated with a high sensitivity membrane (YSI 57776), and a combination glass electrode (Beckman 39532) or in spectrophotometric cuvettes, both provided with rapid stirring and sealed with a perspex plug with a 2 cm long, thin channel filled with the sample, for insertion of microsyringe needles. Mitochondria were suspended in: 130 mM LiCl, 1 mM KCl, 1 mM HEPES, 30 nmol NEM/mg protein, 0.1 μg valinomycin/mg protein, 0.5 μg rotenone/mg protein, pH 7.4.

3. RESULTS

Fig. 1 shows spectrophotometric measurements of

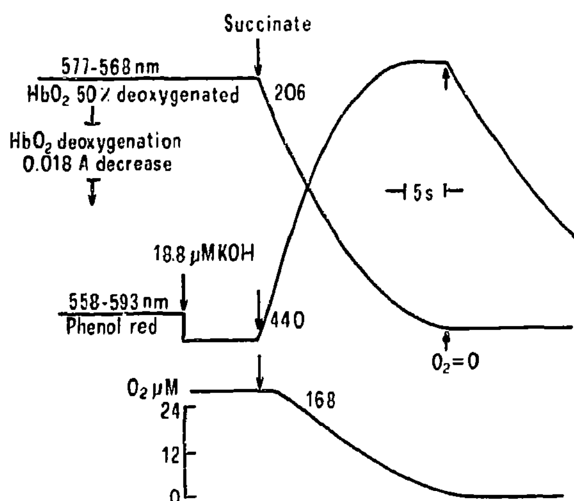

Fig. 1. Measurement of level flow $\leftarrow H^+/e^-$ ratios for succinate respiration in rat liver mitochondria. Mitochondria (2.5 mg protein·ml) were suspended in the reaction medium described in section 2. O_2 was removed from the mitochondrial suspension in the glass cell and spectrophotometric cuvette by gentle argon flow, after which both cells were sealed with the O_2-proof plug. When the O_2 concentration was lowered to 27 µM, and 25 µM HbO_2, added to the suspension in the spectrophotometric cuvette, was 50% deoxygenated, respiration was started by addition of 1 mM succinate. Controls showed that hemoglobin had no effect per se on the initial rate of O_2 consumption measured polarographically. H^+ translocation was measured spectrophotometrically on a sample of the mitochondrial suspension treated used for spectrophotometric measurement of respiration, with the only difference that hemoglobin was replaced with 50 µM phenol red, pH, 7.4, at 25°C. The figures on the O_2 and Hb traces represent initial respiratory rates in equivalents e^-·min·mg protein. Those on the pH traces represent initial rates of H^+ translocation, equivalents H^+·min·mg protein. The measured K_m for O_2 of the Hb preparation sampled used in the experiment was 28 µM and the correlated factor 'f' at 25 µM Hb was 2.1 (see [12,16].

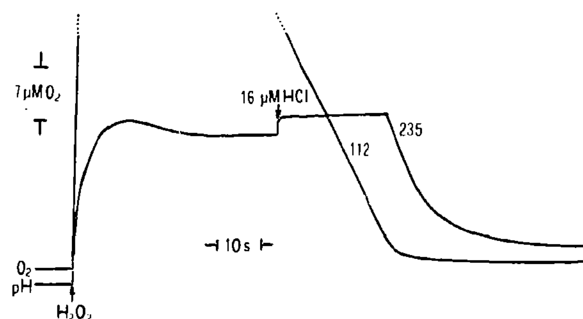

Fig. 2. Measurement of steady-state $\leftarrow H^+/e^-$ ratios for succinate respiration in rat liver mitochondria. Mitochondria (2.5 mg protein·ml) were suspended in the medium described in Section 2 and supplemented with 10 mM succinate. The suspension was left to become anaerobic in a closed glass cell thermostated at 25°C. Once anaerobiosis was reached, 0.1 mg/ml of purified catalase (Boehringer, Mannheim) was added and after 5 min equilibration, respiration was started by addition of 4 µl/ml of 0.2% H_2O_2. The $\leftarrow H^+/e^-$ ratio was calculated form the steady-state e^- flow rate and the initial rate of anaerobic H^+ influx.

respiration and H^+ ejection in rat liver mitochondria supplemented with rotenone and valinomycin plus K^+. Succinate addition to the mitochondrial suspension, when $[O_2]$ had been pre-lowered so as to cause 50% deoxygenation of HbO_2, resulted in an immediate deoxygenation of hemoglobin, from which the initial respiratory rate was calculated as in [12]. It can be noted that the O_2 electrode underestimated the initial respiratory rate. The respiratory burst was accompanied by immediate H^+ translocation which was monitored on separate samples where hemoglobin was replaced by phenol red. The $\leftarrow H^+/e^-$ ratio obtained from initial rates of e^- flow and H^+ release at level-flow was, in this experiment, 2.1. The same ratio was obtained when H^+ release was measured potentiometrically (not shown) (see also [12]).

In the experiment of Fig. 2, mitochondria supplemented with succinate were left to become anaerobic. After 5 min equilibration, respiration was activated by addition of H_2O_2 in the presence of added catalase. An immediate acidification took place which reached a steady-state level in about 1 min, when respiratory rate adjusted itself at a constant steady rate. Upon anaerobiosis immediate alkalinization occurred. At the steady-state the rate of H^+ efflux is equal to that of H^+ influx; the latter is obtained by measurement of the initial rate of anaerobic H^+ influx [13]. The steady-state $\leftarrow H^+/e^-$ ratio amounted, under these conditions, to 2.1.

Fig. 3 presents a comparative analysis of $\leftarrow H^+/e^-$ ratios for H^+ pumping associated to aerobic oxidation of succinate and ascorbate plus TMPD as a function of the respiratory rate. Under level-flow conditions the $\leftarrow H^+/e^-$ ratio for succinate respiration was practically 2 at the higher rates of e^- flow (Fig. 3A, curve a). As the rate of e^- flow was depressed by adding, in different samples, increasing concentrations of malonate, the $\leftarrow H^+/e^-$ ratio first increased up to around 2.8, then further inhibition of e^- flow resulted in progressive decrease of the ratio until at extremely low rates it returned to the value of 2.

Under steady-state conditions (Fig. 3A, curve b) the $\leftarrow H^+/e^-$ ratio exhibited practically the same rate-dependence observed at level-flow. The steady-state $\leftarrow H^+/e^-$ ratios, however did not reach the values found at level-flow for intermediate flow rates. The highest $\leftarrow H^+/e^-$ values observed at the steady-state were around 2.4, and thus significantly lower than the level-flow $\leftarrow H^+/e^-$ ratio of around 2.8 observed at optimal flow rates.

Differently from what was observed for overall electron flow from succinate to O_2, the $\leftarrow H^+/e^-$ ratio measured for electron flow from succinate to ferricyanide (cytochrome *c* reductase span) was constantly 2 over the same range of e^- transfer rates in which succinate respiration exhibited a variable $\leftarrow H^+/e^-$ ratio.

In Fig. 3B the observed $\leftarrow H^+/e^-$ ratios for ascorbate respiration, as a function of the respiratory rate,

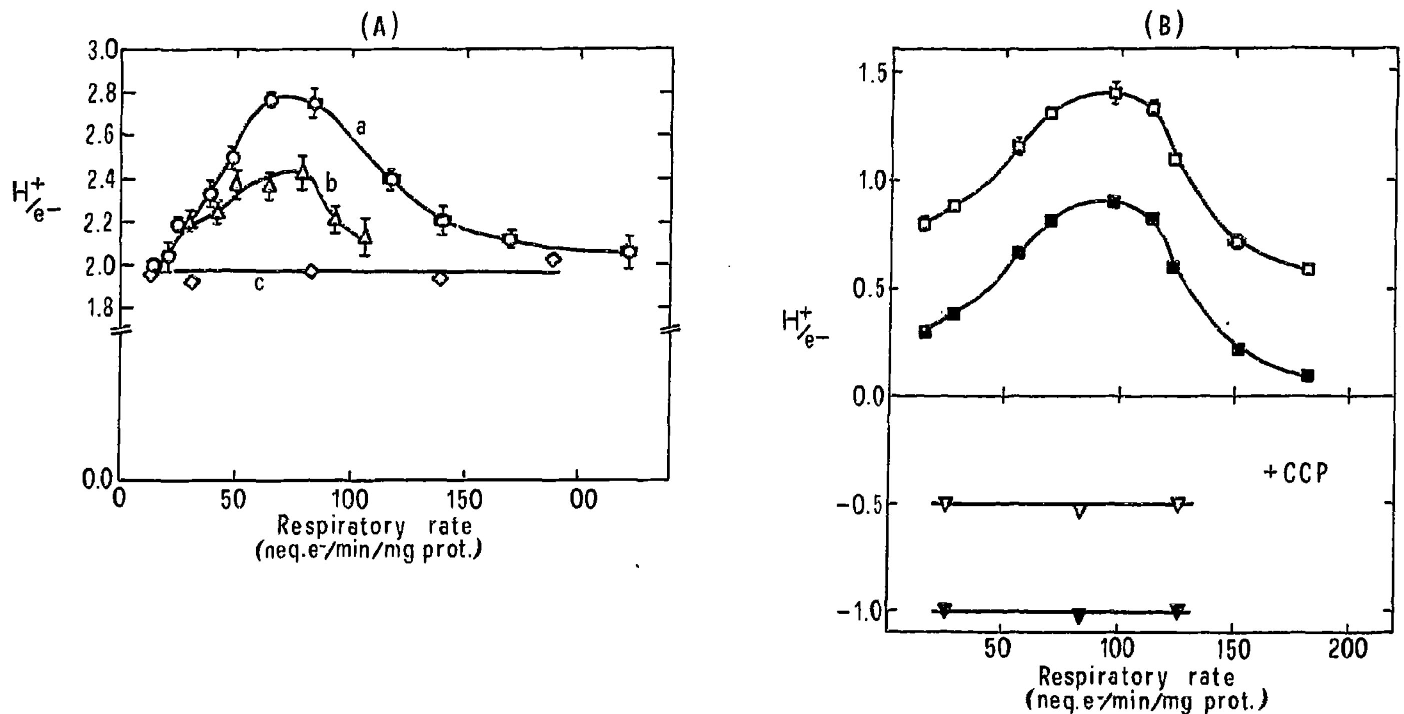

Fig. 3. Analysis of the H^+/e^- ratios for redox linked H^+ translocation as a function of the rate of electron transfer in rat liver mitochondria. *Panel A*. (a) Level flow $\leftarrow H^+/e^-$ ratios for succinate respiration, mean of 11 experiments ± SEM; the respiratory rate was varied by the addition in different samples of increasing amounts of malonate; for other experimental conditions see legend to Fig. 1. (b) Steady-state $\leftarrow H^+/e^-$ ratios for succinate respiration (8 experiments), the respiratory rate was varied by malonate, for other experimental conditions see legend to Fig. 2. (c) Level flow $\leftarrow H^+/e^-$ ratios associated with electron flow from succinate to ferricyanide. (2 experiments). Mitochondria (2.5 mg protein/ml) were suspended in the medium described in Section 2 supplemented with 1 mM KCN. Ferricyanide reduction was measured spectrophotometrically at 420–500 nm; H^+ release was measured on another sample of the same suspension supplemented with 50 μM phenol red. Reduction of ferricyanide and H^+ release were initiated by the addition of 1 mM succinate. The rate of electron flow was varied by malonate. *Panel B*. (□—□) level flow $\leftarrow H^+/e^-$ ratios associated with ascorbate oxidation (4 experiments). Mitochondria (2.5 mg protein/ml) were suspended in the medium described in Section 2 supplemented with 0.05 μg antimycin A/mg protein. The initial rate of H^+ translocation and e^- flow were measured as described in Section 2 and in the legend to Fig. 1. Respiration was activated by addition of 1 mM ascorbate plus various concentrations of TMPD ranging from 5 to 160 μM. For other details see Fig. 1, pH 7.4; 25°C. (▽—▽) H^+/e^- ratios for proton consumption associated with ascorbate oxidation in the presence of 3 μM CCP. The experimental conditions were the same as in the coupled state. (■—■) and (▼—▼) refer to H^+/e^- ratios corrected for the scalar H^+ release contributed by the oxidation of ascorbate to dehydroascorbate. Where not shown the SEM bars fall inside the symbols.

adjusted by varying TMPD concentration, are presented. The $\leftarrow H^+/e^-$ ratio calculated from initial rates at level-flow exhibited a rate dependence similar to that observed for succinate respiration. With ascorbate plus TMPD the $\leftarrow H^+/e^-$ ratios varied from around 0.6 at the highest rates explored, increased up to a maximum value of 1.4 at intermediate e^- flow rate, and then decreased to around 0.8 as the rate of e^- flow was further depressed. The $\leftarrow H^+/e^-$ ratio corrected for the scalar production of 0.5 H^+/e^- associated to oxidation of ascorbate to dehydroascorbate, varied, correspondingly, from 0.1 to 0.90 and back to 0.3. The same rate-dependent changes of the $\leftarrow H^+/e^-$ ratio have been observed in purified cytochrome *c* oxidase reconstituted in liposomes (see accompanying paper [17]). The H^+/e^- ratio for H^+ consumption associated to aerobic oxidation of ascorbate plus TMPD in the presence of CCP was 0.5 (1.0 after correction for the scalar H^+ production associated to oxidation of ascorbate to dehydroascorbate) independent of the respiratory rate.

4. DISCUSSION

The data presented show that in isolated mitochondria the phenomenological $\leftarrow H^+/e^-$ stoichiometry for the cytochrome chain varies under the influence of the respiratory rate and transmembrane $\Delta\mu H^+$.

At level-flow, in the absence of significant $\Delta\mu H^+$ and changes in H^+ conductance, the $\leftarrow H^+/e^-$ ratio for succinate respiration varied from minima of around 2, at extreme high (cf. [12]) and low respiratory rates, to about 3 at intermediate rates. Under the same conditions the $\leftarrow H^+/e^-$ ratio for ascorbate respiration varied from minima of around 0 to 1 at intermediate respiratory rates. The $\leftarrow H^+/e^-$ ratio for electron flow from succinate to ferricyanide was, on the contrary, invariably 2 over all the rate range explored.

The same rate-dependence of the $\leftarrow H^+/e^-$ ratio in the oxidase was observed in the purified bovine heart enzyme reconstituted in liposomes, whilst the $\leftarrow H^+/e^-$ ratio for reconstituted cytochrome *c* reductase remained constant (see [17,18]).

It is, thus, clear that the observed rate-dependence of the $\leftarrow H^+/e^-$ ratio for H^+ pumping by the mitochondrial respiratory chain is associated with electron flow in the oxidase.

Comparison of the $\leftarrow H^+/e^-$ ratios for succinate respiration at level-flow and steady-state shows that the highest ratios attainable at the steady-state, at intermediate flow rates, were significantly lower than those observed for the same rates at level-flow. This provides direct evidence that, besides the flow rate, also the magnitude of $\Delta\mu H^+$ affects the $\leftarrow H^+/e^-$ stoichiometry. Murphy and Brand ([19], however see [20]) have reported that $\Delta\mu H^+$ depressed the charge/e^- ratio in cytochrome oxidase. In their experiments $\Delta\mu H^+$ was lowered by inhibiting steady-state electron flow, thus distinction between effects of $\Delta\mu H^+$ and flow rate could not be made.

The systematic analysis of factors affecting the $\leftarrow H^+/e^-$ stoichiometry for H^+ pumping by cytochrome oxidase which our group has carried out, (see also [17,21]) seems to solve the controversy so far registered for the protonmotive activity of the oxidase [4-8]. The variability of the $\leftarrow H^+/e^-$ stoichiometry documented, provides an explanation for the different values previously observed.

Dependence of the phenomenological $\leftarrow H^+/e^-$ stoichiometry of cytochrome *c* oxidase on flow rate and $\Delta\mu H^+$ seems, in principle, to favour indirect [2,22-24] over direct [1,8] pumping models. Examination of this aspect is, however, beyond the scope of the present paper.

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