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H^+/e^- stoichiometry of mitochondrial cytochrome complexes reconstituted in liposomes

Rate-dependent changes of the stoichiometry in the cytochrome *c* oxidase vesicles

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The H^+/e^- stoichiometry of protonmotive cytochrome *c* oxidase, isolated from bovine heart mitochondria and reconstituted in liposomes, has been determined by making use of direct spectrophotometric measurements of the initial rates of e^- flow and H^+ translocation. It is shown that the $\leftarrow H^+/e^-$ ratio for redox-linked proton ejection by the oxidase varies from around 0 to a maximum of 1 as a function of the rate of overall electron flow in the complex.

Cytochrome *c* oxidase; Cytochrome *c* reductase; H^+ pumping; H^+/e^- stoichiometry; Proteoliposome

1. INTRODUCTION

Reduction of O_2 to H_2O catalyzed by cytochrome *c* oxidase (EC 1.9.3.1) is organized in the membrane [1], so as to directly generate transmembrane $\Delta\mu H^+$ [2]. It has, in addition, been shown that the oxidase of mitochondria and various bacteria pumps H^+ from the inner to the outer aqueous phase [3]. The lack of organic hydrogen carriers in the oxidase has led authors [3–6] to propose indirect pumping mechanisms (cf. [2,7]). These, differently from the direct ligand conduction mechanisms [1,8], predicting fixed output $\leftarrow H^+/e^-$ ratios, can give rise to variable stoichiometries [2,5,6].

The $\leftarrow H^+/e^-$ ratios experimentally determined for cytochrome oxidase, both in native (see accompanying paper, [9]) and artificial membranes, vary from 1 to 0 [3,10–12] (ratios higher than 1 have also been reported [13,14]). $\leftarrow H^+/e^-$ ratios of ≈ 1, observed with the oxidase from bovine heart mitochondria reconstituted in liposomes, were obtained by pulsing the oxidized enzyme with molar excess of ferrocytochrome *c* (or artificial reductants) at neutral pH and high ionic strength (for review see [3,4]). Our group found that, whilst under these conditions the $\leftarrow H^+/e^-$ ratio was indeed 1, changes in the pH and concentration of ferro- and ferri-cytochrome *c*, the presence of divalent cations and different modalities of initiating electron flow, gave $\leftarrow H^+/e^-$ ratios significantly lower than 1 [15].

In this paper, by making use of direct spectrophotometric rate measurements of both e^- flow and H^+ translocation, it is shown that the intrinsic $\leftarrow H^+/e^-$ stoichiometry of redox-linked proton ejection in bovine heart cytochrome oxidase reconstituted in liposomes (COV) varies from around 0 to a maximum of about 1, depending on the actual rate of electron flow in the enzyme. The $\leftarrow H^+/e^-$ stoichiometry in cytochrome *c* reductase vesicles (CRV) is, on the contrary, rate-independent.

Abbreviations: COV, cytochrome *c* oxidase vesicles; CRV, cytochrome *c* reductase vesicles; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; CCP, carbonylcyanide *m*-chlorophenyl-hydrazone; HEPES, *N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid]; EDTA, ethylenediaminetetracetic acid; Hb, deoxyhemoglobin; HbO_2, oxyhemoglobin; $\leftarrow H^+/e^-$, number of H^+ equivalents released from COV or CVR per equivalent e^- transfer.

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2. MATERIALS AND METHODS

Cytochrome *c* oxidase, 10–11 nmol heme $a + a_3$/mg protein [16,17], and cytochrome *c* reductase, 4 nmol cytochrome c_1/mg protein [18,19], were prepared from beef-heart mitochondria. Reconstitution of cytochrome *c* oxidase or cytochrome *c* reductase into phospholipid vesicles (Soybean phospholipids, Sigma) was performed by cholate dialysis [15,20]. More than 85% of both respiratory complexes were incorporated right-side out [15,21]. Respiratory control was higher than 10 for both cytochrome *c* oxidase and cytochrome *c* reductase vesicles.

Cytochromes were measured using the following extinction coefficients: cytochromes $a + a_3$, $\Delta\epsilon_{605-630}$ (red.-ox.) = 14 $mM^{-1} \cdot cm^{-1}$; cytochrome c_1, $\Delta\epsilon_{553-540}$ (red.-ox.) = 17.5 $mM^{-1} \cdot cm^{-1}$; cytochrome *c*, $\Delta\epsilon_{550}$ (red.-ox.) = 21 $mM^{-1} \cdot cm^{-1}$.

H^+ translocation was measured by dual-wavelength spectrophotometry at 558–593 nm with the pH indicator phenol red (see also [9]). Oxygen uptake was measured spectrophotometrically by hemoglobin as in the accompanying paper [9] and in [22].

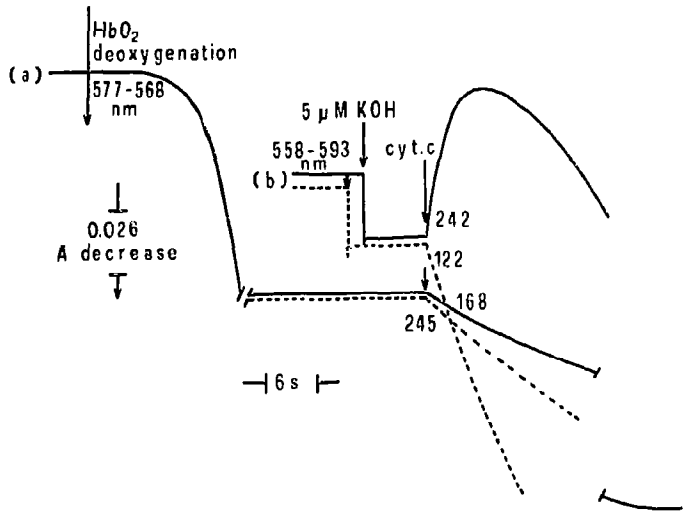

Fig. 1. Measurement of $\leftarrow H^+/e^-$ ratios for redox-linked H^+ translocation in COV. COV (0.35 μM a, a_3) were suspended in the medium described in section 2 and supplemented with 6.5 mM ascorbate plus 50 μM TMPD. The O_2 concentration was lowered by flowing argon onto the surface of the stirred suspension of COV in the spectrophotometric cuvette until recording of absorbance changes at 577–568 nm (trace a) showed that 25 μM added Hb was 50% deoxygenated. The cuvette was then sealed. The Hb absorbance trace showed that no detectable O_2 diffusion occurred in the suspension. The same experimental procedure was followed for measurement of pH changes on a separate sample of the same COV suspension where Hb was substituted with 50 μM phenol red and absorbance changes were monitored at 558–593 nm (trace b). 1 min after HbO_2 was 50% deoxygenated, respiration was started in both samples with the addition of 0.6 μM ferricytochrome *c* (horse heart cytochrome *c*, type VI, Sigma). The numbers on the traces refer to initial rates of e^- transfer to O_2 and H^+ released or consumed (equivalents $e^- \cdot min \cdot ml$ and equivalents $H^+ \cdot min \cdot ml$) respectively. The measured K_m for O_2 of the Hb preparation sample used in the experiment was 37 μM and the correlated factor 'f' at 25 μM Hb was 2.48 (see [22]). When the traces were analysed by Guggenheim plots (0.25 s intervals) the extrapolated initial rates were the same as those reported, obtained drawing the tangent to the initial part of the curve. Dashed traces refer to an experiment where, 3 μM CCP was added. Separate controls showed that at the two wavelength couples, the addition of ferri-cytochrome *c* under the same conditions as the experiments presented but in the absence of both Hb and phenol red did not cause any detectable absorbance change at the two wavelength couples used.

Cytochrome *c* oxidase vesicles (COV) and cytochrome *c* reductase vesicles (CRV) were suspended in100 mM choline-Cl, 5 mM KCl, 0.1 mM choline-EDTA, 2 μg valinomycin/ml, pH 7.4.

3. RESULTS

$\leftarrow H^+/e^-$ ratios for redox-linked H^+ translocation in COV were obtained from spectrophotometric measurements of initial rates of O_2 consumption and H^+ translocation, to overcome problems which may originate from mismatching of electrode performances when electrometric methods are used [23].

In the experiment of Fig. 1, COV were supplemented with ascorbate plus TMPD and valinomycin. The addition of a stoichiometric amount of ferricytochrome *c* to the suspension, whose O_2 concentration had been prelowered by argon flux so as to give 50% deoxygenation of added hemoglobin (cf. [9]), resulted in immediate activation of respiration, as signalled by the absorbance decrease at 577–568 nm (associated with HbO_2 deoxygenation) and H^+ release, monitored by absorbance increase at 558–593 nm in the phenol-red supplemented COV sample (solid curves). The $\leftarrow H^+/e^-$ ratio obtained from the initial rates of H^+ release and e^- flow was 1.44 in this experiment. This, corrected for the scalar production of 0.5 H^+/e^-, associated with oxidation of ascorbate to dehydroascorbate, gives an intrinsic $\leftarrow H^+/e^-$ ratio of 0.94 for H^+-pumping by the oxidase.

In control experiments, the addition of ferricytochrome *c* to ascorbate plus TMPD-supplemented soluble cytochrome oxidase, or COV treated with CCP (see Fig. 1, dashed curves), resulted in direct H^+ consumption at the expected H^+/e^- ratio of 0.5 for aerobic oxidation of ascorbate (correction for scalar H^+ production in the oxidation of ascorbate to dehydroascorbate gives, a net H^+/e^- consumption of 1, see Fig. 2).

In Fig. 2 the results of a systematic analysis of the H^+/e^- stoichiometry for COV and CRV, as a function of the respiratory rate, are presented. When the respiratory rate was varied by changing the concentration of TMPD in COV supplemented with ascorbate, the $\leftarrow H^+/e^-$ ratios showed a bell-shaped dependence on respiratory rate, with values ranging from minima of around 0.80 at the two extremes to a maximum of around 1.4 at intermediate rates (Panel A, curve a). Correction for the scalar contribution of H^+ release associated to ascorbate oxidation gives $\leftarrow H^+/e^-$ ratios, attributable to the oxidase, varying from minima of 0.3 to a maximum of 0.9 (panel B, curve a).

In a parallel set of experiments COV were supplemented with soluble cytochrome *c* reductase and duroquinol as respiratory substrate. When the respiratory rate of COV supplemented with duroquinol was varied by changing the concentration of added soluble cytochrome *c* reductase, the $\leftarrow H^+/e^-$ ratios varied from minima of 1.1–1.2 to a maximum of 1.8 (Fig. 2, panel A, curve b). After correction for scalar production in the oxidation of duroquinol to duroquinone, the resulting curve (Fig. 2, panel B, curve b) was practically similar to the corrected curve for COV supplemented with ascorbate plus TMPD.

In other experiments, the influence of the rate of electron flow on redox-linked proton pumping in cytochrome *c* reductase vesicles (CRV) was also examined. CRV were supplemented with duroquinol and soluble cytochrome *c* oxidase. Respiration and the accompaning H^+ release were initiated by the addition of cytochrome *c* (see [25]). The respiratory activity of CRV was varied with the addition of antimycin A. Redox-linked proton pumping in CRV exhibited, at difference of what observed with COV, a fixed $\leftarrow H^+/e^-$

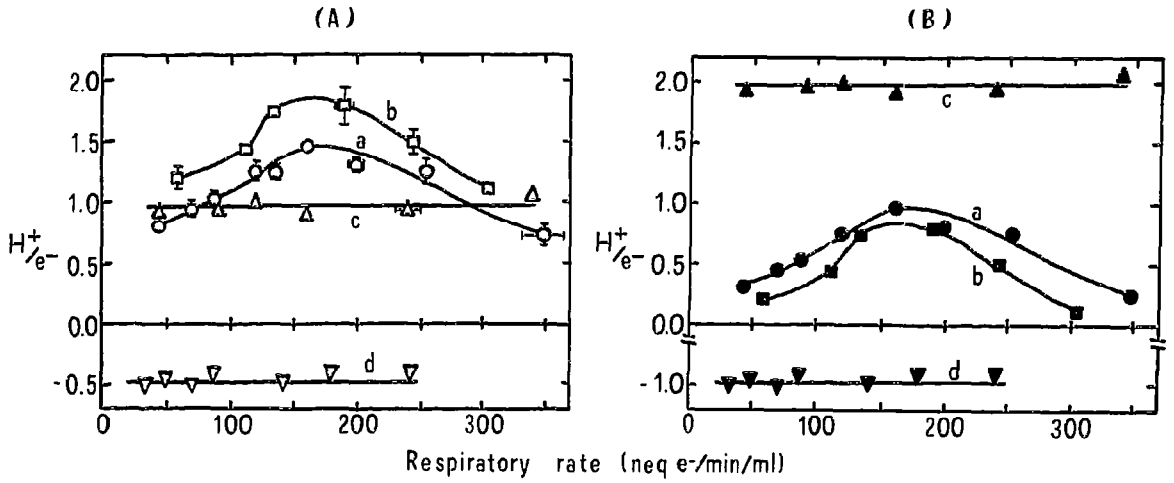

Fig. 2. Analysis of the H^+/e^- ratios for redox-linked H^+ translocation in COV and CRV as a function of the rate of electron transfer. (a) COV supplemented with ascorbate plus TMPD, mean of 8 experiments ±SEM. For experimental conditions see legend to Fig. 1: (d) as (a) plus 3 μM CCP, mean of 8 experiments. The respiratory rate was varied changing the concentration of TMPD from 5 to 400 μM. (b) COV, 0.35 μM aa_3, suspended in the medium described in section 2 and supplemented with soluble cytochrome *c* reductase plus 200 μM duroquinol (mean of 3 experiments). The respiratory rate was varied, changing the concentration of soluble cytochrome *c* reductase (from 0.06 to 0.28 μMi cytochrome c_1). (c) CRV (0.35 μM cytochrome c_1) were suspended in the medium described in section 2 supplemented with soluble cytochrome *c* oxidase (0.35 μM a,a_3) and 200 μM duroquinol. Respiration was started with the addition of 0.6 μM ferricytochrome *c*. For the experimental procedure and other details see legend to Fig. 1 and section 2 (mean of 3 experiments). The respiratory rate was varied by the addition, in different samples, of increasing concentrations of antimycin A up to 0.26 μM. In panel A the H^+/e^- ratios, as directly obtained from the measured initial rates, are presented. The H^+/e^- ratios presented in panel B were obtained by correcting the ratios in panel A for the scalar H^+-release contributed by the oxidation of ascorbate to dehydroascorbate (release of 0.5 H^+/e^-), traces a and d or oxidation of duroquinol to duroquinone (release of 1.0 H^+/e^-), traces b and c. Where not shown, the SEM bars fall inside the symbols. For other experimental details see legend to Fig 1.

ratio of 2.0, which was independent of the respiratory rate (Fig. 2, panel B, curve c).

Control experiments showed that the H^+/e^- ratio for scalar H^+ consumption associated with aerobic ascorbate oxidation by COV supplemented with TMPD and cytochrome *c* was, in the presence of CCP, 0.5 (1, after addition of H^+ produced in the oxidation of ascorbate to dehydroascorbate), independent of the respiratory rate (Fig. 2, panels A and B, curve d). In particular it can be noted that both the H^+/e^- ratio for scalar H^+ consumption with uncoupled COV and the $\leftarrow H^+/e^-$ ratio for H^+ pumping by CRV vesicles did not change over the same range of respiratory rates in which the bell-shaped curve in the $\leftarrow H^+/e^-$ ratio for COV was observed. This eliminates the possibility that the changes observed with COV derived from difficulties in measuring actual rates of e^- flow and H^+ translocation at extreme values, or from problems associated with H^+-leakage in the vesicles.

4. DISCUSSION

The measurements of the $\leftarrow H^+/e^-$ stoichiometry of cytochrome *c* oxidase vesicles reported here have been carried out at level flow, that is, conditions of negligible transmembrane $\Delta\mu H^+$ (see [25]), thus changes in H^+ conductance of the proteoliposomal membrane should not be responsible for the observed changes of the stoichiometry. This is confirmed by the observation that H^+ pumping by cytochrome *c* reductase vesicles exhibits a fixed $\leftarrow H^+/e^-$ ratio of 2, independent of the respiratory rate. It can be concluded that the intrinsic $\leftarrow H^+/e^-$ stoichiometry for proton pumping by cytochrome oxidase varies from around 0 to a maximum of 1 as a consequence of molecular slips in the pump [26] under the influence of the rate of electron flow with a maximum at intermediate respiratory rates (kinetic control of the efficiency of the pump). This situation seems to be different from that exhibited by the pump of cytochrome *c* reductase which does not show any rate-dependent change in the $\leftarrow H^+/e^-$ stoichiometry (see also [9,11,27].

Evidence for possible slippage in the H^+ pump of the oxidase has been obtained in intact mitochondria (accompanying paper [9] and [12]). Molecular slippage in H^+ pumping by cytochrome *c* oxidase, under the influence of kinetic factors [5] and actual pH in the 2 aqueous phases [5,6,15,28] is envisaged by supporters of indirect models of H^+ pumping in the oxidase. Our group has already shown that the phenomenological $\leftarrow H^+/e^-$ stoichiometry of the mitochondrial oxidase reconstituted in liposomes decreases, with reductant pulses, from values around 1, at pH 7.0–7.4 to 0.3 at pH 6.0 (from 0.4 to 0 in oxidant pulses) [15]. The present work provides the first direct experimental demonstration of rate-dependent changes of the phenomenological $\leftarrow H^+/e^-$ stoichiometry of H^+ pumping by mitochondrial cytochrome *c* oxidase in the

purified, reconstituted state. These results seem to favour indirect over direct H^+ pumping models.

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