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Effect of membrane conductance on proton/electron stoichiometry of cytochrome *c* oxidase activity in plant mitochondria

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Proton translocation linked to cytochrome *c* oxidase activity in antimycin-inhibited potato mitochondria has been investigated using the classical oxygen pulse technique. At 25 °C, proton translocation took place with an apparent H^+/e^- ratio of 1.10 ± 0.06 with ascorbate plus *N,N,N′,N′*-tetramethylphenylenediamine (TMPD) and 1.19 ± 0.05 with ascorbate plus ferrocyanide. The decrease in temperature from 25 °C to 5 °C resulted in an increase in membrane viscosity associated with a decrease in the rate constant of the proton back-flow. Under these conditions, the extent of proton ejection was strongly enhanced and the H^+/e^- ratio of ascorbate/TMPD was increased up to 1.73 ± 0.06 and reached 3.65 ± 0.20 for the span succinate-O_2. In the presence of increasing concentrations of the uncoupler CCCP, at 5 °C the H^+/e^- ratio observed or extrapolated at zero time was lowered as the rate of proton back-flow was enhanced. The same relationship was obtained between the H^+/e^- ratio and the rate of proton back-flow after enhancement of proton conductance by either temperature or CCCP. It results that in both cases the calculated H^+/e^- ratio extrapolated to zero time was underestimated. Extrapolation of H^+/e^- ratio in potato mitochondria under conditions of zero proton back-flow provides values which approach 2 for the cytochrome *c* oxidase complex and 4 for the span succinate-O_2.

Introduction

Although proton pumping activity by the cytochrome *c* oxidase (complex IV) has been controversial for a long time [1–5], it is now widely admitted that both proton pumping and charge translocation occur in the terminal segment of respiratory chain of mammalian mitochondria [6–9].

By contrast, in plant mitochondria, data on proton pumping are still poorly documented [10–13] and there is at present no evidence for a direct extrusion of protons from the matrix to the cytosol by the cytochrome *c* oxidase complex in higher plant mitochondria.

In mammalian mitochondria, the H^+/e^- stoichiometry at the level of complex IV ranges between 1 [6,7,9,14] and 2 [15–17] protons extruded by electron transferred. However, there is good agreement on 1 proton per electron with the reconstituted oxidase in liposomes [18–21]. As pointed out by Beavis [22], these discrepancies could be due to the different techniques used to estimate this stoichiometry, including the classical oxygen pulse method [23], steady-state measurements [7], utilisation of fast responding electrodes [8] or pulse-rate methods [9]. From this point of view, rate measurements of proton flux and oxygen uptake in the steady state (leading to a H^+/e^- ratio of 2) are thought to overestimate the real stoichiometry of cytochrome *c* oxidase [5,17].

Beside these technical reasons, several hypotheses have been proposed to explain the different H^+/e^- stoichiometries observed with cytochrome *c* oxidase, such as slippage of the proton pumps [24,25] or proton leakage through the inner mitochondrial membrane [22,26] which could lead to an underestimate of the real stoichiometry in both oxygen and substrate pulse experiments (giving usually an H^+/e^- ratio of 1). Thus,

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; Mops, morpholinopropanesulphonic acid. TMPD, *N,N,N′,N′*-tetramethylphenylenediamine; DPH, diphenylhexatriene.

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the question has arisen as to whether extrapolation to zero time of the amount of ejected protons in the classical oxygen pulse method takes account of the leakage of mitochondrial membranes which is required to approach the real conditions of level flow and the mechanistic stoichiometry of the cytochrome *c* oxidase [16,27].

Recently, a new experimental approach has been developed [28] which avoids these difficulties and provides upper and lower limits for values of H^+/e^- ratios. It has been suggested that the maximal H^+/e^- stoichiometry would be 1.5 for the cytochrome *c* oxidase complex, higher values being considered as thermodynamically impossible [29].

Although cytochrome oxidase of plant mitochondria is generally described as having properties similar to those of the mammalian oxidase, some differences have nevertheless been observed in spectral properties [30] and polypeptide composition [31]. The aim of this study was to examine the extent of the proton pumping by the oxidase of plant mitochondria using the classical oxygen pulse technique, with a special attention focused on proton conductance as a possible source of underestimation of the H^+/e^- ratio. For this purpose, the effects of both temperature and various protonophore concentrations on proton ejection have been particularly investigated. The dependence of proton ejection by the cytochrome *c* oxidase complex of plant mitochondria upon changes of membrane microviscosity with temperature is discussed.

Materials and Methods

Chemicals. Antimycin, valinomycin, rotenone, CCCP and TMPD were purchased from Sigma and were of the highest purity grade. Succinate, ascorbate, ferricyanide, ferrocyanide and TMPD were adjusted to pH 6.5 with potassium hydroxide. Ascorbate, ferrocyanide and TMPD solutions were prepared on the day of experiment.

Preparation of mitochondria. Potato (*Solanum tuberosum* L.) mitochondria were isolated as previously described [32] and purified on discontinuous sucrose gradient. Mitochondria were resuspended in a reaction medium containing: 250 mM sucrose, 100 mM KCl, 5 mM $MgCl_2$, 1 mM Mops and 1 $mg \cdot ml^{-1}$ bovine serum albumin (pH 6.9). The final protein concentration was about 20 $mg \cdot ml^{-1}$ as determined by the biuret.

Oxygen measurements. O_2 uptake was measured polarographically with a Clark-type oxygen electrode (Rank Brothers, U.K.). The chamber (4 ml) was surrounded by a thermostatically controlled water-jacket. Measurements were performed at temperatures ranging between 5 °C and 25 °C. The sample was suspended in the reaction medium and stirred at high speed. For steady-state measurements the oxygen concentration in the reaction medium was 240 μM at 25 °C [33] and 400 μM at 5 °C as determined by the oxidation of external NADH.

pH measurements. Changes in pH were measured at various temperatures in the same vessel chamber using a Beckmann microcombination electrode coupled to a Schott-Gerate digital pHmeter (model CG-809). The electrode was first incubated in 0.1 M HCl for 24 h and maintained in 0.1 M HCl between each pulse experiment. The vessel chamber was closed with a polystyrene plug with thin 4-cm-long channels, filled with the mitochondrial suspension, for insertion of the pH electrode and microsyringe needles. This cell excluded detectable O_2 diffusion.

Oxygen pulse. For oxygen pulse experiments, mitochondria (10 mg) were introduced with the reaction medium to a final volume of 4 ml and a stream of nitrogen was flushed until 95% anaerobiosis was reached. A pH electrode was introduced whose tip was set up near the magnetic stirrer. Inhibitors (1 μM rotenone for succinate oxidation, or 1 μM rotenone + 1 μg antimycin/mg protein for ascorbate oxidation) and ionophore (160 ng valinomycin/mg protein) were introduced 5 min before adding the substrate: ascorbate/TMPD (2.5 mM and 100 μM, respectively), ascorbate/ferrocyanide (0.25 mM and 16 mM, respectively) or 2 mM succinate. Due to the absence of endogenous substrates in purified potato mitochondria, complete anaerobiosis was reached by the addition of substrate after flushing N_2. After a 5 min incubation, the electron acceptor was added: 20 μl of oxygenated water stirred at 25 °C (258 μM) [34] or 20 nmol potassium ferricyanide kept under anaerobiosis. After H^+ decay, when complete equilibrium was obtained, 4 μM CCCP was introduced and mitochondria were kept again in anaerobiosis for 5 more minutes and a new oxygen pulse was initiated. The electrode response was calibrated for each experiment by adding known amounts of previously titrated HCl.

H^+/e^- determination. Net proton ejection was taken at the maximum of the proton pulse linked to the burst of respiration. The amount of ejected protons was then extrapolated from semi-logarithmic plots of the anaerobic decay curve to zero time of oxygen pulse, since the time-course of oxygen pulses was shorter than the response time of the overall experimental device [11]. The extrapolated amounts of protons were then corrected for the protons released from substrate during oxidation and expressed in terms of oxidising equivalents.

Determination of membrane viscosity. Membrane viscosity was assessed by measuring with a Perkin-Elmer LS-5 fluorimeter the fluorescence anisotropy of DPH dye inserted in mitochondrial membranes. Purified mitochondria (40 μg) were introduced in 2 ml suspension medium containing: 250 mM sucrose and 5 mM

Mops (pH 7.0). DPH was introduced to a final concentration of 1 μM. The preparation was excited at 350 nm with a light vertically polarised. The emission intensity at 430 nm was detected through an analyser oriented parallel or perpendicular to the direction of polarisation of the exciting light. Fluorescence anisotropy was calculated directly by the data station after correction for the assymetry of the system [35].

Results

Basically, the determination of H^+/e^- ratio by the oxygen pulse method requires the extrapolation of proton back-diffusion to zero time [23], since the time-course of the oxygen pulse is usually shorter than the response time of the experimental device. In the steady state, the oxidation rates of ascorbate/TMPD and ascorbate/ferrocyanide were 60 ± 15 and 210 ± 5 nmol/min per mg protein, at 25°C, respectively, and 28 ± 4 and 58 ± 5 nmol/min per mg protein at 5°C, i.e., the lower temperature used in further experiments. Based on these steady-state measurements, which underestimate the rate of electron flow occurring in the pulsed state [23], the time-courses of the aerobic phases of the oxygen pulses (10 natom) were less than 0.5 s and 1 s at 25°C and 5°C, respectively.

When oxygen was introduced to an anaerobic suspension of antimycin-inhibited mitochondria in the presence of either ascorbate/ferrocyanide (Fig. 1A) or ascorbate/TMPD (Fig. 1B) a rapid acidification of the external medium (corresponding to a positive H^+/e^- ratio) followed by a slow reequilibration was observed. In the presence of CCCP, a fast alcalinisation followed by a slow reequilibration occurred with both substrates, leading to a net alcalinisation of about -0.5 H^+/e^-. This value resulted from the difference between 1 proton consumed for the formation of water and 0.5 proton released from the oxidation of ascorbate.

With ascorbate/ferrocyanide (Fig. 1A), in the presence of CCCP, extrapolation to zero time of the reequilibration curve led to a net alcalinisation of -0.94 H^+/e^-, indicating that ferrocyanide was mainly oxidised during the short burst of respiration, with only a slight reduction of ascorbate [14,36]. Indeed, if absolutely no protons were produced during the aerobic phase, extrapolation to zero time would give in the presence of CCCP a H^+/e^- ratio of -1 corresponding to the formation of water in the matrix. With ascorbate/TMPD as substrate (Fig. 1B), extrapolation to zero time provided a slightly lower value (-0.71 H^+/e^-). This result is to be expected, since TMPD is partially protonated at neutral pH [14,2]. Thus, net values of extrapolated H^+/e^- ratios determined in the absence of CCCP should be corrected for the amount of protons released by the substrates (ascorbate or TMPD) [2]. These amounts were at 25°C, -0.29 proton per

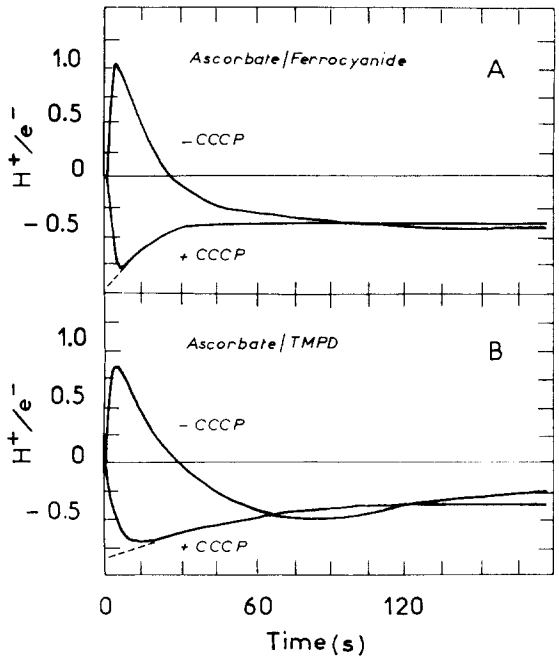

Fig. 1. H^+/e^- in O_2 pulses experiments for antimycin inhibited mitochondria. Anaerobic suspension (4 ml) contained mitochondria (10 mg) in 250 mM sucrose, 100 mM KCl, 5 mM $MgCl_2$, 1 mM Mops, 1 mg·ml^{-1} bovine serum albumin, 1 μM rotenone, 1 μg antimycin/mg protein and 160 ng valinomycin/mg protein at 25°C (pH 6.9). Oxygen pulses were carried out in the presence of 0.25 mM ascorbate plus 16 mM ferrocyanide (A) or 2.5 mM ascorbate plus 100 μM TMPD (B). Proton ejection was started by addition, 5 min after anaerobiosis, of 20 μl of water stirred at 25°C.

electron ($-1 + 0.71$ at zero time) for the couple ascorbate/TMPD, and -0.06 proton per electron ($-1 + 0.94$) for ascorbate/ferrocyanide, respectively.

Table I shows values of H^+/e^- ratios obtained in the presence or in the absence of CCCP, after correction for scalar protons. The mean H^+/e^- ratio was slightly higher for the oxidation of ferrocyanide than for the oxidation of TMPD (1.19 and 1.10, respectively, at 25°C).

In mammalian mitochondria, higher stoichiometries (up to 2 protons per electron) have been reported. One

TABLE I

H^+/e^- ratio at the level of cytochrome c oxidase for antimycin-inhibited potato mitochondria with or without the protonophore CCCP

Net H^+/e^- corresponds to the directly measured stoichiometry and corrected H^+/e^- to the real stoichiometry after collapse of protons from substrate.

Substrate	H^+/e^-		
	+CCCP	−CCCP	
		net	corrected
Ascorbate/TMPD	-0.71 ± 0.04	1.39 ± 0.09	1.10 ± 0.06
Ascorbate/ferrocyanide	-0.94 ± 0.08	1.25 ± 0.20	1.19 ± 0.05

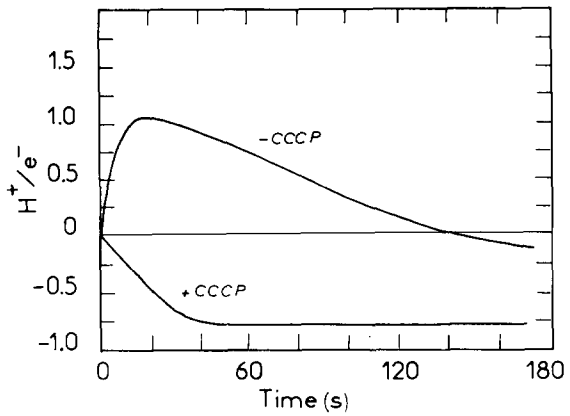

Fig. 2. H^+/e^- during a typical O_2 pulse experiment in potato mitochondria at 5 °C. Oxygen pulses were carried out in the presence of 2.5 mM ascorbate plus 100 μM TMPD. Other conditions were as in Fig. 1.

common explanation is the occurrence of proton leakage which was not taken into account by the extrapolation to zero time. Since a reduction of proton back diffusion as a consequence of a lowering of temperature is expected [16,37], we have measured the H^+/e^- ratios for several spans of the respiratory chain including the complex IV, in a large range of temperature.

Fig. 2 shows an O_2 pulse experiment carried out with ascorbate/TMPD at 5 °C. By comparison with the Fig. 1B, the amount of detectable protons was greatly enhanced and the rate of anaerobic proton back-flow was reduced. In the presence of CCCP, the rate of alcalinisation was considerably lowered. A linear extrapolation provided a value of approx. −0.24 H^+ released per electron transferred at 5 °C (compared to −0.29 at 25 °C, cf. Fig. 1B).

Fig. 3A shows the values of both membrane viscosity and rate constant of proton back-flow determined from the time-course of H^+ decay [11,38] when the temperature was decreased from 35 °C to 5 °C. As the microviscosity was enhanced, the rate constant of proton back-diffusion was strongly decreased from $180 \cdot 10^{-3}$ s^{-1} to $10 \cdot 10^{-3}$ s^{-1}, a value of about $53 \cdot 10^{-3}$ s^{-1} being usually observed at 25 °C. Similar results were obtained with ascorbate/TMPD or succinate as donors and oxygen or ferricyanide as electron acceptors. In both cases (Fig. 3B), a large increase of the extrapolated H^+/e^- ratio was obtained for either succinate and ascorbate/TMPD oxidation. The values of H^+/e^- ratios measured at 5 °C and 25 °C for different spans of the respiratory chain are compared in Table II. The H^+/e^- ratio was 1.73 and 3.65 at 5 °C by comparison with 1.10 and 2.50 at 25 °C for the complex IV and the overall segment succinate-O_2 (complexes II + III + IV) respectively. For the segment succinate-ferricyanide (complexes II + III), very similar results were obtained (H^+/e^- of 1.85). At 25 °C, the H^+/e^- ratio for the overall succinate oxidation appeared underestimated by

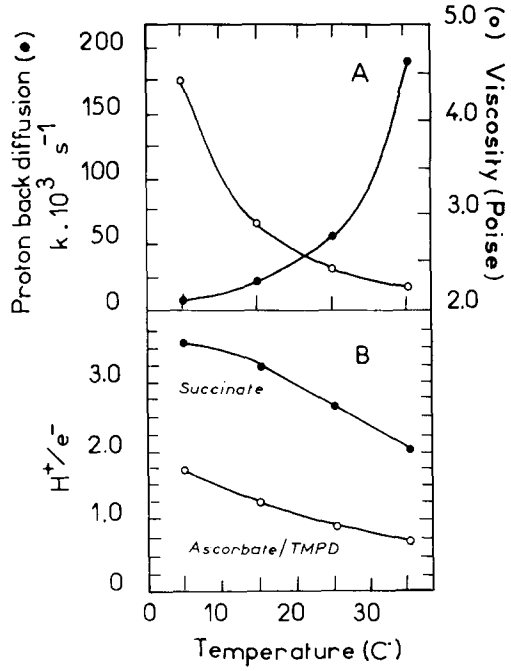

Fig. 3. Influence of temperature on microviscosity and proton back-diffusion (A), and H^+/e^- ratio (B) in potato mitochondria. For (B), oxygen pulses were carried out in the presence of 2 mM succinate (●) (without antimycin) or 2.5 mM ascorbate plus 100 μM TMPD (○). Other conditions were as in Fig. 1.

comparison with the value calculated using the results obtained for complexes II + III and complex IV independently. Similar comparison carried out at 5 °C provides closer value of H^+/e^- ratio for the span succinate-O_2.

As an attempt to analyse the relationship between the rate constant of proton back-flow and the apparent stoichiometry of proton ejection obtained by varying the temperature, the effect of CCCP at low temperature was compared with the temperature effect (Fig. 4). Proton ejection was measured at 5 °C in the presence of low concentrations of protonophore (CCCP) to progres-

TABLE II

Influence of temperature on H^+/e^- ratio for various spans of the respiratory chain in potato mitochondria

Conditions were as in Fig. 1. Pulse experiments were carried out in the presence of: 2 mM succinate as substrate and 20 nmol of ferricyanide as electron acceptor (complexes II + III); ascorbate (2.5 mM) plus TMPD (100 μM) as substrate, antimycin 1 μg/mg protein) and 10 natom oxygen as electron acceptor (complex IV); succinate as substrate and 10 natom oxygen as electron acceptor (complexes II + III + IV).

Temperature °C	H^+/e^-		
	complexes II + III	complex IV	complexes II + III + IV
5	1.88 ± 0.24	1.73 ± 0.06	3.65 ± 0.20
25	1.82 ± 0.13	1.10 ± 0.06	2.50 ± 0.07

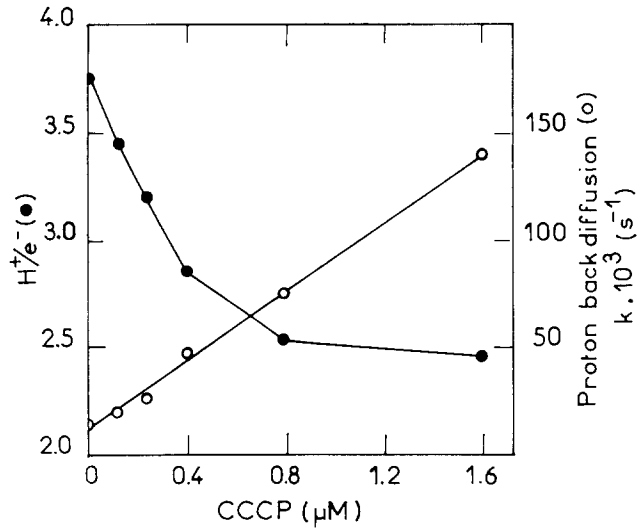

Fig. 4. Influence of various concentrations of the protonophore CCCP on H^+/e^- ratio (●) and rate of proton back diffusion (○) during a typical oxygen pulse experiment carried out at 5 °C in potato mitochondria in the presence of 2 mM succinate. Other conditions were as in Fig. 1 except that antimycin was absent.

sively increase the proton conductance. Both rate constant of proton back-flow and extrapolated values of H^+/e^- were calculated.

Increasing the uncoupler concentration resulted in a progressive decrease in the size of proton pulse linked to succinate oxidation. For CCCP concentrations higher than 1.6 μM the size of the pulse was insufficient to allow accurate determination. As shown in Fig. 4, there was a good linear relationship between the value of the rate constant of proton back-flow which increased with CCCP concentration. By contrast, for CCCP concentrations lower than 0.4 μM, which induced a small increase of the rate constant of proton back-flow, there was a rapid decrease in the values of extrapolated H^+/e^- ratio.

Fig. 5 shows values of H^+/e^- ratio expressed as a function of the rate constant of proton back diffusion, this parameter being modulated either by temperature or by addition of uncoupler (cf. Figs. 3 and 4). In both cases, for succinate oxidation, the H^+/e^- ratio was decreased by a similar way. The extrapolation of H^+/e^- ratio to a rate constant null ($k = 0$) provided a value close to 4, namely a stoichiometry which corresponded to an extrusion of 2 protons per electron at the level of cytochrome *c* oxidase as observed in the case of the oxidation of the couple ascorbate/TMPD (cf. Fig. 5).

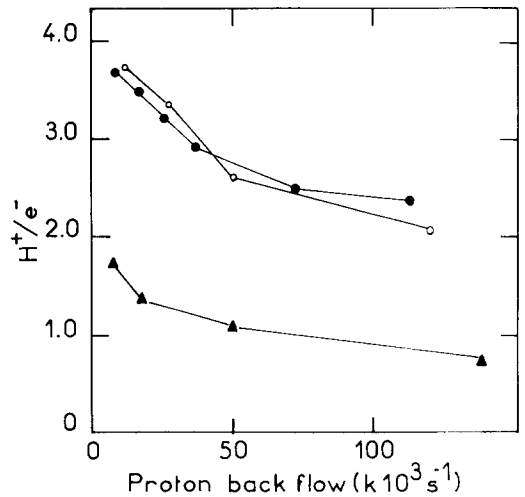

Fig. 5. Relationship between H^+/e^- ratio and proton back diffusion in potato mitochondria during a typical oxygen pulse. ○, when temperature was enhanced from 5 °C to 35 °C and, ●, in the presence of various concentrations of protonophore CCCP (see Fig. 4) in the presence of 2 mM succinate (without antimycin), respectively; ▲, when temperature was enhanced from 5 to 35 °C with 2.5 mM ascorbate plus 100 μM TMPD as substrate. Other conditions were as in Fig. 1.

Discussion

When purified potato mitochondria were introduced in the vessel chamber, absolutely no oxidation took place, even with the high amounts of mitochondrial protein (10 mg) used in oxygen pulse experiments. Only 95% anaerobiosis was reached by flushing N_2 in the suspension medium, and complete anaerobiosis was obtained upon adding exogenous substrates. Consequently, for antimycin-inhibited potato mitochondria, in the presence of valinomycin (plus K^+ as compensating charge), the acidification of the external medium observed during the oxidation of both ascorbate/TMPD and ascorbate/ferrocyanide corresponds to a net H^+ extrusion at the level of the cytochrome *c* oxidase. In this technique, theoretical conditions of level flow are approached by extrapolation of proton back diffusion to zero time [16,27]. Moreover, the net proton production is calculated after correction for scalar protons at 25 °C as at 5 °C, which can be deduced from proton pulse obtained in the presence of CCCP [2,14]. Under these conditions, the net proton production found in potato mitochondria was 1.10 and 1.19 per electron at 25 °C, for the couples ascorbate/TMPD and ascorbate/ferrocyanide, respectively. Such values correspond to the stoichiometry close to 1 proton per electron which is usually obtained by this technique in mammalian mitochondria [9,14].

When temperature was decreased to 5 °C, an increase in the microviscosity of mitochondrial membranes leading to a significant reduction in the rate constant of proton back-flow during oxygen pulse was observed. For a given temperature, similar values were obtained whatever the nature of the donors or acceptors of electrons used in the pulse experiments (i.e., ascorbate or succinate and oxygen or ferricyanide). This suggests that at a given temperature the value of the rate constant of proton back-flow was related to the

proton conductance of the inner membrane as shown by the linear relationship between the value of the rate constant of proton back-flow and the uncoupler concentration.

Both the observed and extrapolated H^+/e^- ratios were strongly increased under conditions of lower membrane conductance. A stoichiometry close to 1.73 proton per electron was found for the cytochrome *c* oxidase complex and 3.65 protons per electron for the whole span of complexes II + III + IV (cf. Table II). Interestingly, the H^+/e^- ratio linked to the complexes II + III only (about 1.9 protons per electron) was not significantly enhanced at low temperature. As a matter of fact, enhanced values of H^+/e^- ratio measured at low temperature could result from a decrease in the H^+ leakage which should not be taken into account by extrapolation to zero time [22,26,37].

Experiments of oxygen pulse were carried out at low and constant temperature in the presence of a protonophore (CCCP) and compared to measurements obtained at different temperatures. The enhancement of proton conductance induced by the protonophore results in a strong decrease in the extrapolated values of H^+/e^- ratios. Indeed, during the aerobic phase of proton pulse, H^+ translocation occurs with a concomitant and significant proton back flow and the amount of extrapolated H^+ depends on the relative rates of H^+ extrusion and H^+ uptake [16,17,26,37] which are differently modulated by temperature. Effectively, when temperature was raised from 5 °C to 25 °C, the oxidation rate (and consequently proton ejection) was enhanced by a factor of 4 (from 50 to 210 nmol O_2/min per mg protein), whereas the value of the rate of proton back-flow was increased by a factor of 10. Thus, the low values of about 1 for H^+/e^- usually found with the classical method of oxygen pulse correspond to an inaccurate estimation of the amount of ejected protons. As the same decrease of stoichiometry was observed by CCCP or increasing temperature, the enhanced value of H^+/e^- ratio obtained by the oxygen pulse method at low temperature is likely to be related to a decrease in membrane conductance [37].

The maximal and theoretical values of H^+/e^- stoichiometry determined by the oxygen pulse technique are clearly underestimated by the proton back-flow occurring during the aerobic phase and higher values can be obtained only when the leakage of membrane is known or abolished [17,26,28,29]. For this purpose, values of H^+/e^- have been tentatively extrapolated at zero conductance, providing values close to 4 protons per electron for the span succinate/O_2 and 2 protons per electron at the level of the cytochrome *c* oxidase. Based on thermodynamic considerations, it has recently been suggested that, in the steady state, the maximal value of H^+/e^- should not exceed 1.5 [29]. However, it can be speculated that such a limitation does not apply under conditions of oxygen pulses in which the rate of proton back-flow is close to zero (condition of level flow) [16,27].

It has been shown that during pulse experiments the proton back-flow depends on the extent of the transmembrane ΔpH and the buffer capacity of the matrix [15,20,26]. During the overall succinate oxidation the transmembrane proton gradient is lower with ferricyanide as electron acceptor than with oxygen, since in the former case no protons disappear in the matrix for the formation of water. Consequently, the H^+/e^- ratio for the span succinate-ferricyanide was close to the theoretical value of 2 protons per electron usually observed in mammalian mitochondria [39–42] and this value was unaffected at low temperature. It can be mentioned that the decrease in H^+/e^- ratio with the magnitude of $\Delta\mu H^+$ was not found for the complex III when ferricyanide was used as substrate, by contrast with the cytochrome *c* oxidase complex [42].

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