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Proton/Electron Stoichiometry of Mitochondrial bc_1 Complex. Influence of pH and Transmembrane ΔpH

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The effect of pH and transmembrane ΔpH on the efficiency of the proton pump of the mitochondrial bc_1 complex both *in situ* and in the reconstituted state was studied. In both cases the H^+/e^- ratio for vectorial proton translocation by the bc_1 complex respiring at the steady state, under conditions in which the transmembrane pH difference (ΔpH) represents the only component of the proton motive force (Δp), was significantly lower than that measured under level flow conditions. The latter amounts, at neutral pH, to 1 (2 including the scalar H^+ release). In the reconstituted system steady-state ΔpH was modulated by changing the intravesicular buffer as well as the intra/extra-liposomal pH. Under these conditions the H^+/e^- ratio varied inversely with the ΔpH. The data presented show that ΔpH exerts a critical control on the proton pump of the bc_1 complex. Increasing the external pH above neutrality caused a decrease of the level flow H^+/e^- ratio. This effect is explained in terms of proton/electron linkage in *b* cytochromes.

KEY WORDS: bc_1 complex; mitochondria; transmembrane pH difference; cytochromes; H^+/e^- stoichiometry.

INTRODUCTION

Electron flow in the bc_1 complex in the mitochondrial membrane is compulsorily linked to vectorial proton translocation from the matrix (N phase) to the intermembrane space (P phase). Under level flow conditions (i.e., under conditions of negligible transmembrane Δp), two protons are vectorially translocated for every two electrons transferred from ubiquinol to cytochrome *c* by the complex, either in the native membrane or in the reconstituted proteoliposomal complex (Leung and Hinkle, 1975; Papa *et al.*, 1980, Lorusso *et al.*, 1983). Two additional protons derive formally from the scalar oxidation of the substrate quinol by cytochrome *c*, thus accounting for the overall H^+/e^- stoichiometry of 2. Under level flow conditions a decrease of the phenomenological H^+/e^- ratio was reported following chemical modification of the protein complex either *in situ* or in isolated form by DCCD[2] (Degli Esposti *et al.*, 1982; Lorusso *et al.*, 1983; Nalecz *et al.*, 1983; Clejan *et al.*, 1984). A decoupling effect was also observed upon controlled proteolytic cleavage of polypeptide subunits of the isolated bc_1 complex (Lorusso *et al.*, 1989); Cocco *et al.*, 1991).

Uncoupler titration experiments on the proton pumping activity of liposome-reconstituted bc_1 complex (Cocco *et al.*, 1992) showed that respiration-dependent transmembrane pH difference lowers the steady-state H^+/e^- stoichiometry (see also Rich and Heathcote, 1983).

In this paper the control exerted by ΔpH on proton pumping by the reconstituted bc_1 complex is

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[2] Abbreviations: DCCD: dicyclohexylcarbodiimide; TMPD: *NNN'N'*-tetramethyl-*p*-phenylenediamine; ΔpH: transmembrane pH gradient; $\Delta\Psi$: transmembrane electrical potential gradient; Δp: transmembrane protonmotive force; pH_{in}: inner-phase pH; pH_{out}: outer-phase pH; CCCP: carbonyl cyanide *m*-chlorophenylhydrazone; NEM: *N*-ethylmaleimide; DQH_2: duroquinol; DCIP: 2,6-dichloroindophenol.

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studied by two different approaches. Furthermore, a new procedure is introduced showing that also in the native mitochondrial membrane the steady-state H^+/e^- ratio is markedly lower than that measured under level flow conditions. Experiments are also presented showing that the H^+/e^- ratio, under both level flow and steady-state conditions, is influenced by the actual pH in the aqueous phases.

MATERIALS AND METHODS

Preparation of Cytochrome *c* Reductase and Cytochrome *c* Oxidase Complexes

The cytochrome *c* reductase and cytochrome *c* oxidase complexes were isolated from bovine-heart mitochondria according to Rieske (1967) and Errede *et al.* (1978), respectively.

Preparation of Mitochondria

Rat liver mitochondria were isolated as described by Pedersen (1977). Protein content was determined by the biuret method (Gornall *et al.*, 1949) using bovine serum albumin as standard. The content of bc_1 complex in the mitochondrial preparation was estimated spectrophotometrically (see also Estabrook and Holowinsky, 1961).

Preparation of bc_1 vesicles

Reconstitution of bc_1 complex into phospholipid vesicles was performed by the cholate dialysis method of Leung and Hinkle (1975) and basically as reported by Cocco *et al* (1992), with a sonication mixture consisting of 100 mM potassium-Hepes (pH 7.4), 56 mM KCl, and 2% potassium cholate. Different conditions used were: (i) 5 mM potassium-Hepes (pH 7.4), 97 mM KCl; (ii) 100 mM potassium-Hepes at pH varying from 7 to 8.5 with varying concentrations of KCl to a final 100 mM K^+ concentration. The first and the second (overnight) dialysis steps were performed against the same sonication medium with the omission of cholate. The dialysis medium in the last two hours contained 1 mM potassium-Hepes and 100 mM KCl. In all the dialysis media the pH was that of the sonication mixtures. Where required, the potassium activity of the bc_1 vesicle suspension was lowered by performing the last dialysis step against 1 mM potassium-Hepes, 1 mM KCl, and 0.2 M sucrose.

Measurement of Protonmotive Activity in bc_1 Vesicles

Vectorial proton translocation in bc_1 complex was essentially measured as described by Cocco *et al.* (1992). bc_1 vesicles (final concentration 0.8 μM cytochrome c_1) were suspended in 1.5 ml of 2 mM Hepes (pH 7.4) and 100 mM KCl, containing 2 μg valinomycin, 0.4 μM soluble cytochrome oxidase, and 300 μM duroquinol. The reaction was started by the addition of 0.4 μM cytochrome *c*. H^+ translocation was measured potentiometrically with a combination glass electrode. Oxygen uptake was simultaneously measured with a Clark oxygen electrode (4004 YSI, Yellow Spring, Ohio) coated with a high-sensitivity membrane (YSI 57776) in a thermostatically controlled (25°C) all-glass cell also housing the glass electrode. This procedure allows measurement of pure vectorial proton translocation since protons released in the oxidation of quinol are taken up in the reduction of oxygen to H_2O by the soluble oxidase added in the external medium. The H^+/e^- ratio at level flow was calculated from the initial rate of proton translocation and electron transfer ensuing upon addition of cytochrome *c*. The H^+/e^- ratio at the steady state was calculated from the initial rate of proton back-flow ensuring upon interruption of respiration by antimycin (1.2 μM) and the steady-state oxygen consumption rate exhibited just before respiration was stopped.

Measurement of Redox and Protonmotive Activities in Mitochondria

Level flow H^+/e^- ratio for succinate respiration was determined by measuring proton translocation potentiometrically and oxygen uptake spectrophotometrically following the deoxygenation of human hemoglobin (Papa *et al.*, 1980b, 1991) in a sealed cuvette also housing the glass electrode. The correction factor f for the hemoglobin used was 1.71. For the steady-state experiments oxygen uptake was measured electrometrically. Proton translocation associated to electron flow from succinate to ferricyanide was measured as described by Papa *et al.* (1980a). Level-flow H^+/e^- ratio associated to ascorbate (+TMPD) oxidation was measured as described by Papa *et al.* (1991).

Measurement of Passive Proton Permeability

Proton permeability in bc_1 vesicles and rat liver mitochondria was measured as described by Lorusso *et al.* (1983) and Brown and Brand (1986).

Fluorescence Measurements

Respiration-dependent ΔpH generation was measured by pyranine-containing bc_1 vesicles as described by Cocco *et al.* (1992). bc_1 vesicles (final concentration 0.15 μM cytochrome c_1) were suspended in the same medium used for sonication and dialysis containing 0.1 μM soluble cytochrome oxidase, 200 μM duroquinol, and 2 μg valinomycin. The reaction was started by the addition of 0.12 μM ferricytochrome *c*. The final volume was 3.5 ml. Changes of pyranine fluorescence were monitored with a Perkin-Elmer 650 fluorescence detector. The excitation and emission wavelengths were 460 and 520 nm, respectively.

The membrane potential generated by succinate-supported respiration in mitochondria was monitored following the fluorescence quenching of externally added 5 μM safranin (Singh and Nicholls, 1985; see also Akerman and Wikstrom, 1976) at the excitation and emission wavelengths of 525 and 575 nm, respectively. Rat liver mitochondria (0.25 mg/ml) were suspended in a mixture containing 130 mM LiCl, 1 mM potassium-EGTA, 1 mM Hepes (pH 7.4), 30 nmol NEM/mg protein, 0.5 μg rotenone/mg protein, and 1.6 μg oligomycin/mg protein. Respiration was started by the addition of 10 mM succinate.

CHEMICALS

Horse heart cytochrome *c* (type VI), antimycin, valinomycin, nigericin, CCCP, and safranin O were from Sigma Chemical Co.; catalase was from Boehringer Mannhein; duroquinol was from K. & K. Laboratories; pyranine was from Eastmann Kodak Co. All other reagents were of the highest purity grade commercially available.

RESULTS

Effect of Transmembrane ΔpH

In the experiment shown in Table I, H^+/e^- ratios at level flow and steady-state respiration in bc_1 vesicles prepared with 5 mM and 100 mM potassium-Hepes respectively were measured. Vesicles highly buffered inside showed H^+/e^- ratios for vectorial proton translocation of 1.0 and 0.32 for level flow and steady-state conditions, respectively. The level flow H^+/e^- ratio did not vary when the intravesicular buffer capacity was lowered, whereas the steady-state H^+/e^- ratio significantly decreased. Measurement of respiration-dependent steady-state transmembrane pH difference, by following the fluorescence signal of proteoliposome entrapped pyranine, showed that the steady-state H^+/e^- ratio varied inversely with regard to ΔpH.

Table I. Influence of Intraliposomal Buffer Capacity of H^+/e^- Ratio for Vectorial Proton Translocation and Transmembrane ΔpH in bc_1 Vesicles[a]

	Level flow	Steady state	
Internal buffer	H^+/e^-	H^+/e^-	ΔpH
Hepes 5 mM	1.00 (±0.02)	0.25 (±0.01)	0.45 (±0.01)
Hepes 100 mM	0.99 (±0.02)	0.32 (±0.02)	0.37 (±0.02)

[a] H^+/e^- ratios and transmembrane ΔpH were measured as described under Materials and Methods. The data reported represent the mean of four experiments.

Figure 1 presents data from three sets of experiments in which the H^+/e^- ratio was measured as a function of pH. The indicated pH values refer to both the intravesicular space and the external medium. This condition is advisable for correct measurement of respiratory transmembrane ΔpH. As the pH was increased to above 7.5, the level flow H^+/e^- ratio significantly decreased, whereas the steady-state H^+/e^- ratio increased. Steady-state ΔpH, measured under the same conditions, appeared to decrease at alkaline pHs. At pH values higher than 8.0 the signal of pyranine was too small to be reliable. The values of

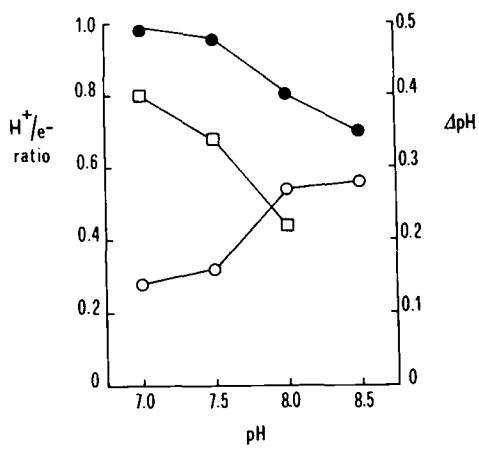

Fig. 1. pH-dependence of H^+/e^- ratios and transmembrane ΔpH in bc_1 vesicles. bc_1 vesicles prepared at different intraliposomal pH were suspended in the reaction mixture whose pH was equal to that of the intravesicular space. The experimental procedures for the measurement of H^+/e^- ratios and transmembrane ΔpH are those reported under Materials and Methods. (●), level flow H^+/e^- ratio; (○), steady-state H^+/e^- ratio; (□), transmembrane ΔpH.

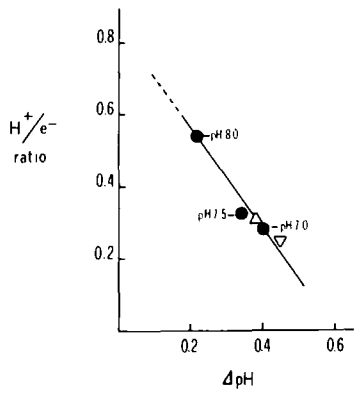

Fig. 2. Relationship between steady-state ΔpH and H^+/e^- ratio in bc_1 vesicles. Steady-state H^+/e^- ratios and the coresponding ΔpH values are those from experiments reported in Table I and Fig. 1 and refer to vesicle preparation having internal buffer of 100 (△) and 5 (▽) MM Hepes (pH 7.4) or intraliposomal pH ranging from 7.0 to 8.0 (●).

steady-state H^+/e^- ratio as a function of transmembrane ΔpH obtained in the experiments shown in Table I and Fig. 1 and reported in Fig. 2 and show a linear inverse correlation between steady-state H^+/e^- ratio and ΔpH. The resulting straight line is superimposable to that obtained previously (Cocco *et al.*, 1992) by uncoupler titration of vectorial proton translocation in bc_1 vesicles.

The measurement of steady-state H^+/e^- ratio for vectorial proton translocation in the bc_1 complex in the native mitochondrial membrane was carried out as shown in Fig. 3. The level flow H^+/e^- ratio for succinate respiration was preliminarily measured by pulsing the aerobic suspension of rat liver mitochondria with succinate (Fig. 3A). Oxygen uptake was measured spectrophotometrically following deoxygenation of added oxyhemoglobin, and proton translocation was measured potentiometrically. From the initial rates of oxygen consumption and proton translocation, an H^+/e^- ratio of 2.6 was determined (Papa *et al.*, 1991). In the experiment of Fig. 3B, mitochondrial suspension was allowed to become anaerobic by succinate respiration. Oxygen was then delivered in the medium by the addition of H_2O_2 in the presence of added catalase. The onset of respiration was accompanied by acidification of the external medium which attained a steady-state level in about 60 sec. Steady-state H^+/e^- ratio was calculated from the initial rate of proton back-flow ensuing upon anaerobiosis and the steady-state rate of oxygen consumption. In the experiment shown here a value of 1.9 was calculated, which is significantly lower than the level flow H^+/e^- ratio. Antimycin was added to the same sample of anaerobic suspension of mitochondria (1.1 mol per mol of bc_1 complex). This led to 95% inhi-bition of succinate-supported respiration. Cholate-solubilized bc_1 complex isolated from bovine heart was then added and, after 15 min incubation, respiration was activated by a second H_2O_2 pulse. The amount of soluble bc_1 complex added (0.1–0.16 nmol/mg mitochondrial protein) was adjusted in order to reconstitute a succinate oxidase activity as close as possible to that of the control. Under these conditions, the overall steady-state H^+/e^- ratio for proton translocation that can be ascribed only to the activity of the cytochrome oxidase complex amounted to 1.4. Subtraction of this value from that obtained for the initial steady-state H^+/e^- ratio gives an H^+/e^- ratio of 0.5 for vectorial proton translocation by the bc_1 complex. Statistical evaluation of a set of six experiments gave a value of 0.51 ± 0.03. Separate control experiments showed that: (i) Soluble bc_1 Soluble bc_1 complex itself, at the concentrations used here, did not show any appreciable succinate-ferricyanide reductase or ascorbate (+TMPD) oxidase activity, even in the presence of added cytochrome *c*. (ii) 15-min incubation of soluble bc_1 complex with antimycin-inhibition mitochondria was required to reconstitute full succinate oxidase activity. This relatively long time of incubation did not cause damage of the mitochondrial membrane as revealed by the finding that electron transfer from succinate to ferricyanide measured in intact mitochondria, after 15 min incubation at 25°C, gave, at pH 7.2, the expected H^+/e^- ratio of 2. (iii) The H^+/e^- ratio for electron transfer from succinate to ferricyanide was 1 when antimycin-inhibited mitochondrial were reconstituted with soluble bc_1 complex. This shows that externally added soluble bc_1 complex, which underwent co-precipitation with mitochondria at $8.000 \times g$ upon 15-min incubation, while reconstituting the succinate oxidase activity, did not reconstitute the proton pump activity at the second site. (iv) The proton pumping activity of the mitochondrial cytochrome *c* oxidase associated with the oxidation of ascorbate was not influenced by the presence of externally added soluble bc_1 complex.

The approach included here thus shows that the steady-state H^+/e^- ratio for the vectorial proton translocation by the bc_1 complex in the native membrane is, as already observed with the reconstituted enzyme, considerably lower than that measured under level flow conditions. Separate controls

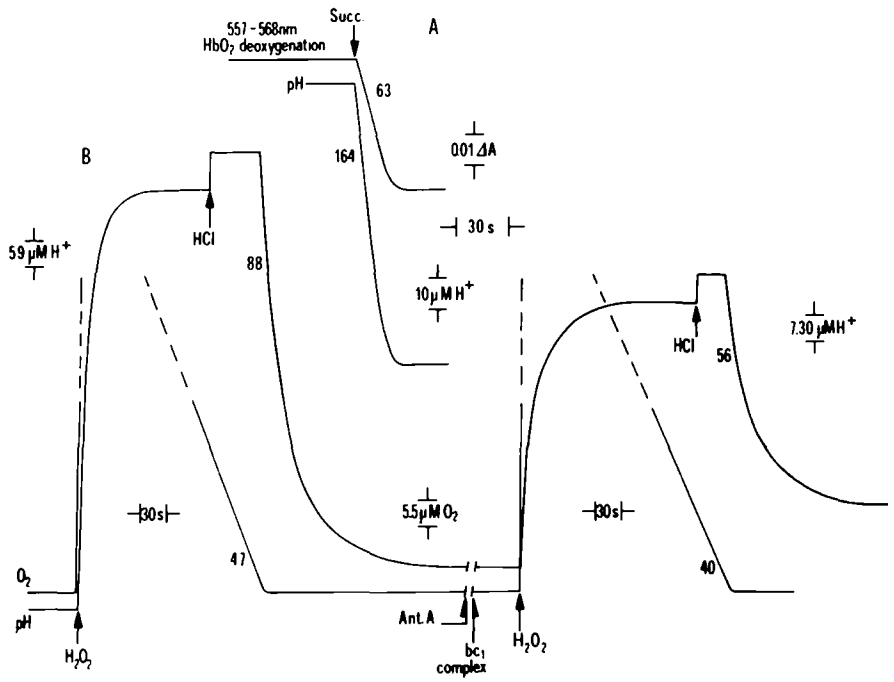

Fig. 3. H^+/e^- ratio for proton translocation associated to succinate respiration in mitochondria. Rat liver mitochondria (2.5 mg protein/ml) were suspended in the reaction mixture consisting of 130 mM LiCl, 1 mM potassium-EGTA, 1 mM Hepes (pH 7.4), 30 nmol NEM/mg protein, 0.1 μg valinomycin/mg protein, 0.5 μg rotenone/mg protein, and 1.6 μg oligomycin/mg protein. In experiment A mitochondrial suspension was supplemented with 20 μM hemoglobin. The reaction was started by the addition of 1 mM succinate. In experiment B the mitochondrial suspension was supplemented with 0.1 mg/ml of purified catalase. 10 mM succinate was added and the suspension was allowed to become anaerobic. After 5 min incubation, respiration was activated by addition of 5 μl of 0.2% H_2O_2. The steady-state rate of proton translocation was calculated from the initial rate of proton back-flow ensuing upon anaerobiosis. Subsequent additions were: 1.1 mol antimycin/mol mitochondrial bc_1 complex followed, after 3 min, by 0.15 nmol/mg mitochondrial protein of soluble bc_1 complex. After 15 min a second 5 μl of 0.2% H_2O_2 pulse was given to the anaerobic mitochondrial suspension. Figures on the traces represent rates of electron flow and proton translocation as $nmol \cdot min^{-1} \cdot mg\ protein^{-1}$. For other experimental conditions and details see under Materials and Methods.

showed that the respiration-dependent transmembrane potential, as detected by the fluorescence signal of safranin, appeared to be completely collapsed by the concentration of valinomycin ($+K^+$) used, indicating that ΔpH was the only component of Δp at the steady state.

Outer Phase pH Influences the Proton Pump of the bc_1 Complex

As shown in Fig. 1, pH increase of intra- and extra-liposomal space caused a decrease of the level-flow H^+/e^- ratio, an increase of the steady-state H^+/e^- ratio, and a decrease of transmembrane ΔpH. It was then attempted to discriminate between pH_{in} and pH_{out} effects.

In the experiment shown in Fig. 4 bc_1 vesicles prepared with the intravesicular pH of 7.0 (A) and 8.0 (B), were exposed to external media whose pH varied from 6.5 to 8.5. Independently of the internal pH, the level-flow H^+/e^- ratio decreased as the external pH was increased above neutrality, whereas the steady-state H^+/e^- ratio increased. The rate of electron transfer was, on the other hand, almost unaffected by the pH increase.

The decrease of the level-flow H^+/e^- ratio observed under these conditions was not due to an increase of proton leakage through the liposomal membrane as a consequence of the exposure of the vesicles to alkaline pH values. Experiments on passive proton diffusion into the vesicles driven by

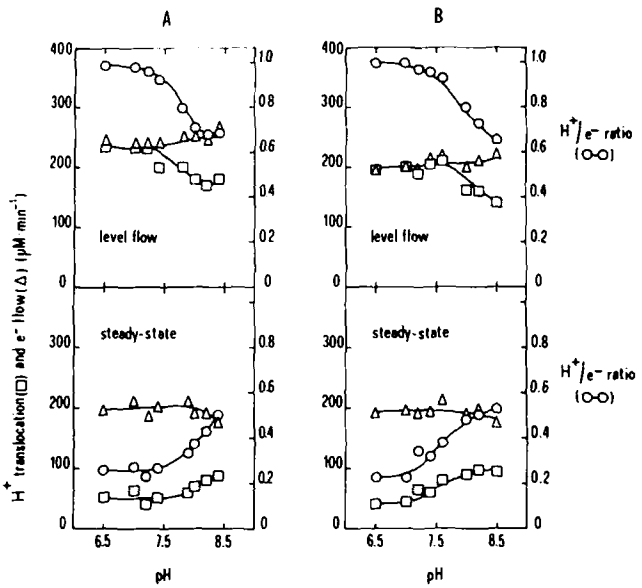

Fig. 4. Influence of extraliposomal pH on H^+/e^- ratio in bc_1 vesicles. bc_1 vesicles at intraliposomal pH of 7.0 (A) and 8.0 (B) (see under Materials and Methods) were suspended in the reaction mixture described under Materials and Methods, at the pH values indicated in the figure. Under conditions of imposed bulk phase pH difference, a small proton equilibration across the membrane occurred before the enzymatic reaction started. This proton flux was taken into account for the estimation of proton release and uptake after the electron transfer reaction was started and stopped, respectively. (△) rates of electron flow; (□) rates of proton translocation; (○) H^+/e^- ratios.

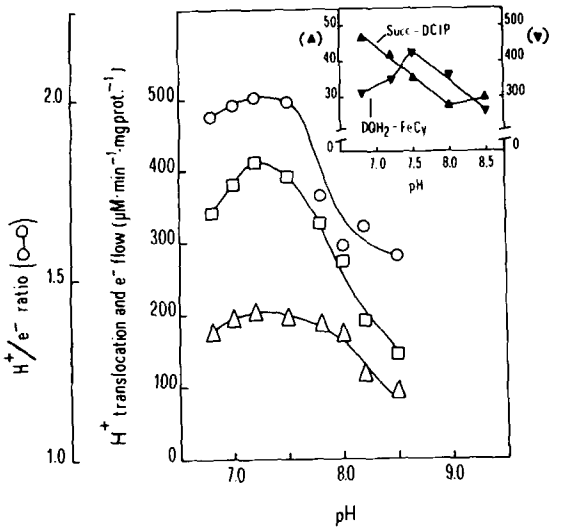

Fig. 5. pH-dependence of level-flow H^+/e^- ratio associated to succinate-ferricyanide reductase activity in rat liver mitochondria. Mitochondria (2.5 mg protein/ml) were suspended in the reaction mixture described in the legend to Fig. 3 supplemented with 1 mM KCN. Ferricyanide reduction was followed spectrophotometrically at 420–500 nm. 100 μM ferricyanide was added in 25-μM aliquots. The first aliquots of ferricyanide underwent partial reduction by endogenous reductants. The absorbance changes caused by the last two aliquots of ferricyanide, which did not undergo further reduction, were equivalent and were used to convert the absorbance decrease caused by succinate addition into nmol of ferricyanide reduced. Proton translocation was followed potentiometrically in the same spectrophotometric cuvette. The reaction was initiated by the addition of 1 mM succinate. (△) rates of electron flow; (□) rates of proton translocation; (○) H^+/e^- ratios. Antimycin-sensitive duroquinol-ferricyanide reductase activity (DQH_2-FeCy) (▼) was measured as described for succinate-ferricyanide reductase activity measurement, with 100 μM duroquinol as substrate. Succinate-coenzyme Q reductase (Succ-DCIP) (▲) was assayed spectrophotometrically by measuring the rate of reduction of 2,6-dichloroindophenol (DCIP) at 600 nm. Mitochondria (0.1 mg protein/ml) were suspended in the reaction mixture described in the legent to Fig. 3 supplemented with 1 mM KCN and 50 μM dichloroindophenol. The reaction was started by the addition of 16 nM succinate.

valinomycin mediated potassium diffusion potential showed that the first-order kinetic constant for proton uptake was, for the vesicles prepared at pH 7.0 and suspended in a medium at same pH, four times higher than that measured with vesicles prepared and suspended at pH 8.0. The total extent of proton uptake, measured in the presence of 2 μM CCCP, was at pH 8.0 even higher than that measured at pH 7.0 (not shown).

The pH dependence of the level-flow H^+/e^- ratio for succinate-supported ferricyanide reduction in KCN-inhibited rat liver mitochondria was also examined (Fig. 5). Electron transfer and proton translocation both showed a bell-shape dependence as a function of the extramitochondrial pH changes. This is likely due to the pH dependence of the succinate-quinone reductase and the quinol-ferricyanide reductase activities (see inset to Fig. 5). The level flow H^+/e^- ratio, which was almost constantly 2 until pH 7.5, decreased at more alkaline values. Separate experiments in which the succinate-ferricyanide reductase activity was inhibited by malonate titration showed that the H^+/e^- ratio was independent of the rate of electron transfer (see also Papa *et al.*, 1991). Similarly to what was observed in the bc_1 vesicles, also the proton passive permeability of the mitochondrial membrane was at pH 8.0 lower than that measured at pH 7.0 (not shown).

DISCUSSION

Conflicting reports have appeared on the influence of Δp on the H^+/e^- ratio for proton translocation by

the bc_1 complex at the steady-state respiration (Rich and Heathcote, 1983; Murphy and Brand, 1988; Brown, 1989; Hafner and Brand, 1991; Luvisetto *et al.*, 1991). Most of the reported experiments were, however, carried out in the presence of nigericin which clamped the ΔpH component of the protonmotive force to near zero, so that the influence of ΔpH *per se* was overlooked (see, however, Brown, 1989). Our group has produced data showing that the H^+/e^- ratio for proton pumping by the bc_1 complex reconstituted in liposomes decreases, at the steady-state respiration, to values well below 1 (Cocco *et al.*, 1992). By acting on the leak properties of the vesicles with subsaturating concentrations of the uncoupler CCCP or by introducing in the medium bovine serum albumin, which reduces passive proton leakage in the mitochondrial membrane (Luvisetto *et al.*, 1991), a linear inverse correlation between the steady-state H^+/e^- ratio and ΔpH was found. In this paper such a correlation is substantiated by two independent approaches, that is, change of the intraliposomal buffering capacity and variation of the pH in the two aqueous phases. It is furthermore shown that also in mitochondria the steady-state H^+/e^- ratio is considerably lower than the level-flow H^+/e^- ratio, thus confirming that, also in the native membrane, transmembrane ΔpH depresses the efficiency of the proton pump of the bc_1 complex at the steady-state. The value of 0.5 found in mitochondria for the steady-state H^+/e^- ratio is somewhat higher than that found under these conditions in the liposomal system in spite of the higher value of ΔpH estimated in mitochondria (Papa *et al.*, 1981, 1983b; Brown and Brand, 1985). A possible explanation of this discrepancy is that ΔpH in proteoliposomes could be underestimated due to the inherent system of measurement. Calibration of fluorescence changes of liposomal pyranine is, in fact, done by adding externally alkali, in the presence of both valinomycin and nigericin, to all the vesicles, including those not incorporating the enzyme (Wrigglesworth *et al.*, 1990).

As a mechanism for ΔpH control on the proton pump of the bc_1 complex at steady-state respiration, we suggest that a highly alkaline pH generated at the catalytic coupling site, which is likely to be deeply buried in the membrane, can depress protonation of the semiquinone/quinone couple from the inner aqueous phase, thus causing decoupling of the pump.

The possibility that the pH generated by redox-linked proton pumping at the coupling domain in the membrane is much more alkaline than that indicated by bulk phase measurement of ΔpH is supported by the pH dependence experiments shown in Fig. 4. A bulk-phase ΔpH of more than one unit imposed by suspending bc_1 vesicles with the internal pH adjusted to 8 into media with neutral or slightly acidic pH did not appear to limit the uptake of protons and their access into the input proton channel at the N side of the membrane, as revealed by the H^+/e^- stoicheiometry of 1 for vectorial proton translocation obtained under these conditions.

Consistent with the view that protonation of the coupling center of the pump from the inner aqueous phase represents the critical rate-limiting step in the presence of the respiration-dependent ΔpH is the observation that in inside-out submitochondrial particles, where the protonation site is in equilibrium with the external medium, the steady-state H^+/e^- ratio for succinate oxidation approaches, at slightly acidic pHs, the value of 3, in the presence of NaSCN which can collapse both the ΔΨ (electrophoretic uptake of SCN^-) and ΔpH components (H_{in}^+/Na_{out}^+ exchange) of Δp (Papa *et al.*, 1973).

The experiments shown in Figs. 4 and 5 indicate that both in bc_1 vesicles and in mitochondria, increasing values of the external pH above neutrality cause a decrease of the level-flow H^+/e^- ratio. This effect can be explained in terms of either (i) dismutation of semiquinone radicals at alkaline pHs (Mitchell, 1982) or (ii) linkage between electron transfer at the metal centers and protolytic events in the apoprotein of *b* cytochromes at pH values above neutrality (redox Bohr effects). Due to the localization of cytochrome *b*-566 at the positive side of the membrane (Papa *et al.*, 1981; Ohnishi *et al.*, 1989) and of cytochrome *b*-562 toward the negative side (Konstantinov and Popova, 1987), transmembrane electron flow from *b*-566 at the P side to *b*-562 toward the N side of the membrane would consist, at alkaline pH values, of an effective hydrogen transfer whose direction is opposite to that of the respiratory proton pump (Papa *et al.*, 1981, 1983a). The decrease of proton translocation caused by external alkalinity and enhancement of the inner buffering power would cause a decrease of the steady-state transmembrane ΔpH, resulting in enhancement of the steady-state H^+/e^- ratio to almost the value observed, at alkaline pHs, under level flow conditions (Figs. 1 and 4).

In conclusion, at the steady-state respiration the efficiency of the proton pump of the bc_1 complex, and of the whole mitochondrial cytochrome system, is

apparently depressed by the chemical component (ΔpH) of the protonmotive force. Utilization of ΔpH by anion translocators (Mitchell, 1966; Chappell, 1968; Papa *et al.*, 1970), the proton/monovalent cation antiport (Mitchell and Moyle, 1969; Douglas and Cockrell, 1974; Papa *et al.*, 1983b), and the ATP synthase can, however, counteract the decoupling effect of ΔpH by keeping it at values compatible with a good efficiency of the pump.

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