Journal of Bioenergetics and Biomembranes, Vol. 29, No. 1, 1997

Original Article

Steady-State Proton Translocation in Bovine Heart Mitochondrial bc_1 Complex Reconstituted into Liposomes

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Received May 6, 1996; accepted June 8, 1996

The effect of different anions on the steady-state proton translocation in bovine bc_1 complex reconstituted in liposomes was studied. The H^+/e^- ratio for vectorial proton translocation is at the steady state definitely lower than that measured at level flow, (0.3 vs. 1.0). The presence of azide or arachidonate at micro- and submicromolar concentrations, respectively, gave a substantial reactivation of the proton pumping activity at the steady state, without any appreciable effect on respiration-dependent transmembrane pH difference. Addition of azide to turning-over bc_1 vesicles also caused a transition of *b* cytochromes toward oxidation. The results are discussed in terms of possible involvement of an acidic residue in the protonation of the semiquinone/quinol couple at the N side of the membrane.

KEY WORDS: bc_1 complex; mitochondria; cytochromes; transmembrane pH difference; H^+/e^- ratio; decoupling; azide; arachidonate.

INTRODUCTION

The mitochondrial bc_1 complex (ubiquinol-cytochrome *c* reductase, complex III) is the middle part of the respiratory chain and catalyzes the transfer of reducing equivalents from ubiquinol to ferricytochrome *c*. Coupled to the electron transfer, the complex translocates protons from the matrix (N phase) into the intermembrane space (P phase). Under level-flow conditions (i.e., under conditions of negligible transmembrane protonmotive force) one proton is vectorially translocated from the N to the P phase for every electron transferred by the complex either *in situ* or reconstituted in liposomes (Leung and Hinkle, 1975; Papa *et al.*, 1980; Lorusso *et al.*, 1983). An additional scalar proton, deriving formally from the oxidation of quinol by ferricytochrome *c* is released at the P site of the membrane and accounts for the overall H^+/e^- ratio of 2. A decrease of the H^+/e^- ratio, under level-flow conditions, was first observed after reaction of the bc_1 complex with DCCD[2] (Degli Esposti *et al.*, 1982; Lorusso *et al.*, 1983; Nalecz *et al.*, 1983; Clejan *et al.*, 1984). The polypeptide subunit whose carboxyl residue(s) undergoes DCCD modification is, however, still under question (see Hassinen and Vuokila, 1993, for review). Subsequently a similar effect, referred to as the decoupling effect, was found to be produced by limited proteolysis of the polypeptide subunits of isolated bc_1 complex (Lorusso *et al.*, 1989; Cocco *et al.*, 1991). More recently a partial decoupling of the bc_1 complex has been shown to be caused by the point mutation Gly 137 → Glu in cytochrome *b* of *S. cerevisiae* (Bruel *et al.*, 1995). Our group has shown that the H^+/e^- ratio for vectorial proton translocation by the bc_1 complex incorporated in liposomes is, at the steady state, significantly lower than that measured under level flow conditions (0.3 vs. 1.0) (Cocco *et al.*, 1992; Lorusso *et al.*, 1995). Since these experiments were carried out in the presence of valinomycin

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[2] ABBREVIATIONS: DCCD, dicyclohexylcarbodiimide; Δp, protonmotive force; ΔpH, transmembrane pH gradient; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DQH_2, duroquinol.

0145-479X/97/0200-0081$12.50/0

(+ K^+), so that the transmembrane pH difference (ΔpH) was the only component of Δp, the conclusion was drawn that transmembrane ΔpH exerts a control on the proton pumping activity of the bc_1 complex under conditions of steady-state respiration. Any experimental condition leading to a decrease of transmembrane ΔpH did cause, in fact, a definite increase of the H^+/e^- ratio. Importantly, the control exerted by ΔpH on the proton pumping activity appears to be operating also with the complex in the native membrane (Lorusso *et al.*, 1995).

In this paper we show that weak acids like azide and arachidonate reverse to a substantial extent the decoupling effect exerted by ΔpH on the proton pump of the bc_1 complex, without affecting the transmembrane ΔpH. The results obtained indicate that the weak acids favor the access of protons from the N side of the membrane into the input proton channel in the bc_1 complex, thus mimicking the role played by specific residues of apoproteins in the mechanism of the redox proton pump.

MATERIALS AND METHODS

Preparation of the 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 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. The first dialysis step was performed against the same sonication medium without cholate; the second (overnight) step was performed against 10 mM potassium-Hepes (pH 7.4) and 96 mM KCl. The dialysis medium in the last two hours contained 1 mM potassium-Hepes (pH 7.4) and 99.6 mM KCl. The pyranine-entrapped vesicles were prepared as described by Cocco *et al.* (1992).

Treatment of bc_1 Vesicles with Anions

bc_1 vesicles, at the concentration indicated below, were suspended in the reaction mixture also containing increasing concentrations of azide, cyanate, and thiocyanate. After 2 min incubation, the reaction was started. Arachidonate was instead incubated with aliquots of vesicle suspension for 15 min. The vesicles were then transferred into reaction mixture also containing the same arachidonate concentration.

Measurement of Protonmotive Activity

Level-flow and steady-state proton translocation in bc_1 vesicles was measured as described by Lorusso *et al.* (1995). bc_1 vesicles (final concentration 0.8 μM cytochrome c_1) were suspended in 1.5 ml of 2 mM potassium-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. 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 backflow ensuing upon interruption of respiration by antimycin (1.2 μM), and the steady-state oxygen consumption rate exhibited just before respiration was stopped.

Fluorescence Measurements

Respiration-dependent ΔpH generation was measured in 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*. Final volume 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.

Spectral Analysis

Spectral analysis in oxidized and dithionite-reduced bc_1 vesicles were performed according to von Jagow *et al.* (1981) with a Perkin-Elmer lambda 5 split-beam spectrophotometer. The suspension mixture contained 0.1 M potassium-Hepes (pH 7.4), 10 mM KCl, 1% (w/v) Tween-80, and 1 mM KCN.

Spectrophotometric Determination of Redox Changes of the Cytochromes

Oxidoreduction of *b* and c_1 cytochromes was followed, at 25°C, with a double-beam dual-wavelength spectrophotometer (Johnson Research Foundation, Philadelphia), at the wavelength couples 562–575 and 566–575 nm for *b* cytochromes, and 552–540 nm for cytochrome c_1. bc_1 vesicles (0.8 μM cytochrome c_1) were suspended in a medium containing 1 mM potassium-Hepes (pH 7.4), 0.1 M KCl, 0.1 μM soluble cytochrome *c* oxidase, and 0.2 μM cytochrome *c*. The steady-state respiration was started by the addition of 200 μM duroquinol.

Chemicals

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

RESULTS

The effect of azide on the electron transfer and proton translocation in bc_1 vesicles under conditions of steady-state respiration is shown in Fig. 1. Micromolar concentrations of azide, owing to its inhibitory effect on the cytochrome *c* oxidase added in the reaction medium to sustain steady-state respiration, progressively depressed the rate of electron transfer, while the rate of proton translocation was unaffected or even stimulated in the range 20–60 μM (Fig. 1A). As a

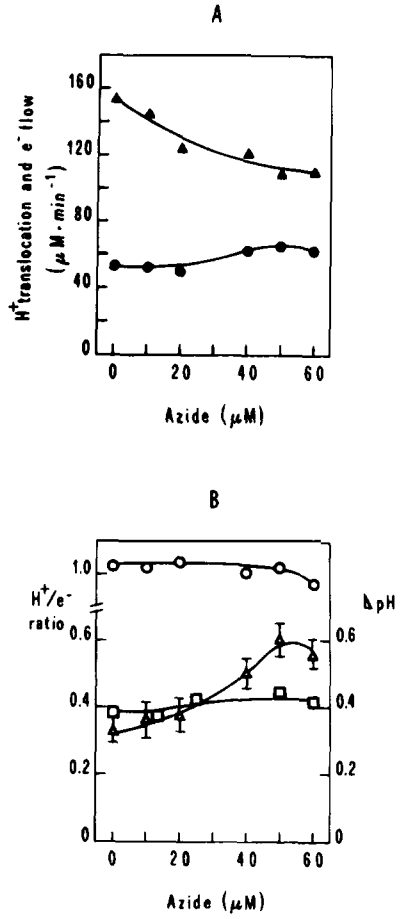

Fig. 1. Effect of azide on proton translocation and electron transfer in bc_1 vesicles. The experimental procedures for the determination of the H^+/e^- ratios and transmembrane ΔpH are described in Materials and Methods. Steady-state proton translocation (●) and electron transfer (▲) activities. (○) level-flow H^+/e^- ratio; (△), steady-state H^+/e^- ratio; (□), transmembrane ΔpH. The values reported are means of eight experiments. S.D. is only reported for steady-state H^+/e^- ratio.

consequence the steady-state H^+/e^- ratio increased from a value of 0.34 in the control to 0.6 at 50 μM azide concentration (Fig. 1B). Direct measurements on the same bc_1 vesicles showed that: (i) the level-flow H^+/e^- ratio (Fig. 1B) for vectorial proton translocation was costantly 1 in the presence of azide, (ii) the transmembrane pH difference set up by respiration was not affected or was even increased by azide; the recoupling effect of azide was, thus, obtained at constant ΔpH. A recoupling effect was also exhibited by other weak acids. Arachidonate at submicromolar concentrations stimulated the steady-state proton translocation under conditions where electron transfer was unaffected (Fig. 2A). The steady-state H^+/e^- ratio was

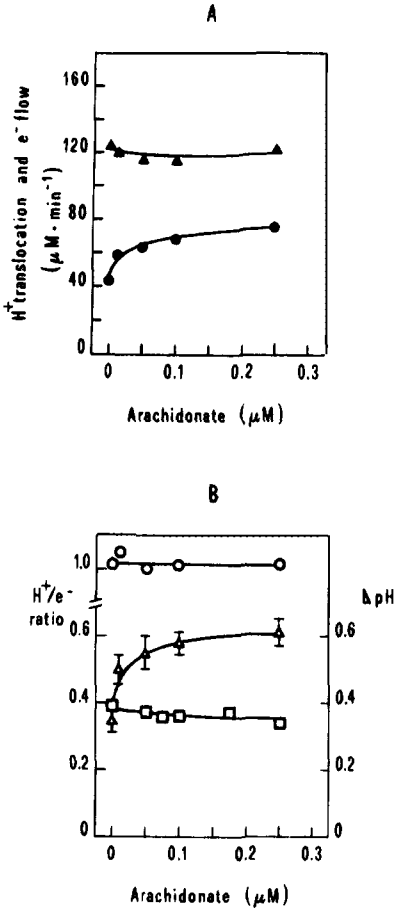

Fig. 2. Effect of arachidonate on proton translocation and electron transfer in bc_1 vesicles. The experimental procedures for the determination of the H^+/e^- ratios and transmembrane ΔpH are described in Materials and Methods. Steady-state proton translocation (●) and electron transfer (▲) activities. (○) level-flow H^+/e^- ratio; (△), steady-state H^+/e^- ratio; (□), transmembrane ΔpH. The values reported are means of eight experiments. S.D. is only reported for steady-state H^+/e^- ratio.

consequently increased. The measured ΔpH was practically constant over all the range of arachidonate concentrations examined (Fig. 2B).

The apparent affinity constant K_a (the concentration at which half maximal increase of the steady-state H^+/e^- ratio occurred) and the extent of stimulation of the H^+/e^- ratio by different anions are reported in Table I. Anions, with pKs around 4, were able to reactivate the proton pump activity. Thiocyanate, on the contrary, did not have any effect up to 10 mM concentration. Among the anions tested, the lipophylic arachidonate showed the lowest K_a value as compared to the water-soluble acids.

Table I. Apparent Affinity Constant, K_a of Different Anions in the Reactivation of ΔpH-Depressed Proton Pump in bc_1 Vesicles[a]

	pK	K_a (μM)	H^+/e^- ratio (% stimulation)
Azide	4.5	33	76
Arachidonate	4.8	0.011	74
Cyanate	3.7	2.3	60
Thiocyanate	0.85	No effect	

[a] For experimental conditions see legend to Figs. 1 and 2 and Materials and Methods. pK values refer to the dissociation constant of the corresponding acids.

Cocco *et al.* (1992) have previously shown that subsaturating concentrations of CCCP enhanced the steady-state H^+/e^- ratio from around 0.3 in the control to values almost double (see also Fig. 3). This effect was correlated with reduction by the uncoupler of the transmembrane pH difference. The experiment reported in Fig. 3 shows that the effects elicited by either CCCP or anions are additive. Their combination allows, in fact, a full reactivation of the proton pump activity of the bc_1 complex at the steady-state.

Effect of Azide on the Steady-State Reduction of Cytochromes

The steady-state reduction levels of b and c_1 cytochromes are reported in Table II. As previously

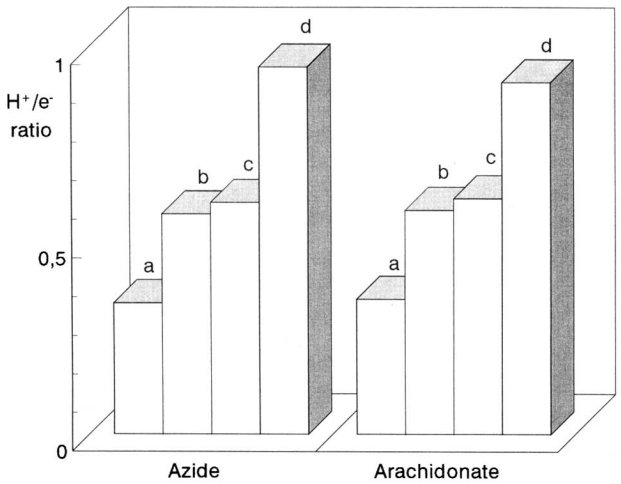

Fig. 3. Full reactivation of the steady-state proton pump of bc_1 vesicles by a combination of anions and CCCP. For the experimental conditions see legends to Figs 1 and 2 and Materials and Methods. (a) control (b) 6 nM CCCP; (c) 50 μM azide or 0.25 μM arachidonate; (d) azide or arachidonate *plus* CCCP.

Table II. Steady-State Redox Transitions of *b* and c_1 Cytochromes upon Addition of Valinomycin and Azide to Turning-over bc_1 Vesicles[a]

	Reduction of					
Additions	*b*-566 (%)	ΔE_h (mV)	*b*-562 (%)	ΔE_h (mV)	c_1 (%)	ΔE_h (mV)
DQH_2	58		72		59	
DQH_2 + val	39	+20	68	+5.0	81	−28
DQH_2 + val + azide	29	+12	64	+4.6	85	−7
DQH_2 + val + azide + CCCP	20	+13	59	+5.5	85	0

[a] bc_1 vesicles were suspended in the reaction medium also containing soluble cytochrome *c* oxidase and traces of cytochrome *c*. The respiration was started by the addition of 200 μM duroquinol. Where indicated, 0.5 μg/ml valinomycin, 50 μM azide, and 6 nM CCCP were added. For other experimental conditions and details see Materials and Methods. The absorbance changes measured at 562–575 and 566–575 nm were used to determine the respective percentage reduction of the individual hemes *b*-566 and *b*-562 (Papa *et al.*, 1981). ΔE_h values refer to the difference in the redox level of cytochromes measured after and before the addition of valinomycin, azide, and CCCP. The values reported represent the mean of four experiments.

reported (Papa *et al.*,, 1983), valinomycin addition to bc_1 vesicles turning over at the steady state caused a transition of both *b*-566 and *b*-562 toward oxidation and transition of cytochrome c_1 toward reduction. The transition of *b*-566 is definitely larger than that of *b*-562 (see Table II). These effects have to be attributed to the control exerted by the respiration-dependent membrane potential on the electron transfer pathway from *b*-566 located at the positive side of the membrane (Papa *et al.*, 1981; Ohnishi *et al.*, 1989) to *b*-562 located toward the negative side (Konstantinov and Popova, 1987), which in turn delivers electron to semiquinone/quinol couple in the quinol pocket. Addition of azide (as well as cyanate, not shown) in the presence of valinomycin caused further oxidation of *b* cytochromes, mostly of *b*-566 (see Table II) and reduction of cytochrome c_1. A similar effect was elicited by azide, although to a lesser extent, even in the absence of valinomycin (not shown). Subsequent addition of CCCP caused a further steady-state oxidation of *b* cytochromes, while cytochrome c_1 was unaffected. The effects elicited by either azide or CCCP on the redox level of *b* cytochromes were independent of the order of their additions to the suspension of turning-over vesicles (not shown). Separate controls showed that azide did not cause any shift in the absorption spectrum of cytochromes neither oxidized nor reduced (not shown).

DISCUSSION

Several reports indicate that the proton pump of mitochondrial bc_1 complex is susceptible to decoupling from the electron transfer activity. These include experiments carried out in the covalently modified bc_1 complex (Degli Esposti *et al.*, 1982; Lorusso *et al.*, 1983; Nalecz *et al.*, 1983; Clejan *et al.*, 1984; Lorusso *et al.*, 1989; Cocco *et al.*, 1991), as well as in cytochrome *b*-mutated yeast enzyme (Bruel *et al.*, 1995).

The control exerted by the protonmotive force on the proton-pumping activity of the cytochrome system has been studied by several groups although with conflicting results (Murphy and Brand, 1988; Brown, 1989; Hafner and Brand, 1991; Luvisetto *et al.*, 1991; Brand *et al.*, 1994; Canton *et al.*, 1995). Our group has provided evidence showing that respiration-dependent transmembrane ΔpH lowers the steady-state H^+/e^- ratio for vectorial proton translocation through the bc_1 complex (Cocco *et al.*, 1992; Lorusso *et al.*, 1995; see also Rich and Heathcote, 1983). In this paper we show that the ΔpH-depressed steady-state proton pumping activity can be substantially reactivated by azide (or arachidonate) at constant ΔpH, and fully reactivated by a combination of these anions and subsaturating concentrations of the uncoupler CCCP. These results rule out the possibility that the lowering of the H^+/e^- ratio we observed under various conditions would represent an experimental artifact or result from difficulties in measuring the proton translocation process in the bc_1 complex at the steady-state respiration.

As a mechanism for the ΔpH control on the steady-state proton pump, it was suggested that a highly alkaline pH generated at the catalytic coupling site (the quinone reduction site) in protonic equilibrium with the N-phase can depress protonation of the semiquinone/quinol couple from this phase, with decoupling of the pump (Lorusso *et al.*, 1995). It is

conceivable that residue(s) in the apoproteins can mediate proton transfer from the inner aqueous phase to the catalytic center (Papa *et al.*, 1983,1994). The protonation of such residue(s), particularly in the presence of a transmembrane ΔpH (alkaline pH at the N side), would represent a critical event in the vectorial proton translocation process.

The weak acids used in the experiments presented here may mimic these amino acid residue(s) in facilitating the protonation of the semiquinone/quinol couple at the catalytic coupling site, thus acting as protein internal protonophores. This situation would be analogous to that proposed by Tittor *et al.* (1989) who found that a defective proton pump in point-mutated bacteriorhodopsin (Asp96 → Asn) was fully reactivated by azide and other anions of weak acids whose p*K*s range between 3.7 and 4.5. Asp96 in the native protein has been shown to occur in the protonated form (Braiman *et al.*, 1988; Gerwert *et al.*, 1989). Azide has also been found to accelerate proton transfer processes leading to formation of quinol, which were severely limited in AspL213 → Asn mutated reaction centers from *Rb. sphaeroides* (Takahashi and Wraight, 1991; Paddock *et al.*, 1994).

Protonation of the semiquinone anion by weak acids would favor acceptance of electrons from cytochrome *b* to form the quinol. This is supported by the observed transition toward oxidation, induced by azide, of *b* cytochromes in turning over vesicles. Due to the rapid redox equilibrium between *b*-562, which is the ultimate reductant of semiquinone, and *b*-566, the oxidation of the latter becomes greater (Table II).

The present results would indicate an involvement of an acidic residue at the input proton channel on the N side of the membrane. The acidic residues whose modification by DCCD results in decoupling of the proton pump have been suggested to belong to either the cytochrome *b* of yeast mitochondria (Beattie, 1993) or to subunit IX (the 8-kDa subunit) of bovine heart bc_1 complex (Lorusso *et al.*, 1983; Degli Esposti *et al.*, 1983). The suggested DCCD-binding acidic residues have, however, been proposed to be located in polypeptide apolar regions lying at the P side of the membrane (Palmer *et al.*, 1993; Beattie, 1993; Borchart *et al.*, 1985), and thus may be only involved in the release of protons upon oxidation of quinol. A good candidate for the acidic residue at the N side might, on the contrary, be an aspartic residue (Asp229) belonging to helix E according to the 8-helix model for cytochrome *b* (Rao and Argos, 1986; Crofts *et al.*, 1987; Brasseur, 1988) and located at the N side of the membrane. This is a fully conserved residue among several eukaryotic species, including man, beef, and yeast (Degli Esposti *et al.*, 1993). This residue corresponds to Asp252 of the cytochrome *b* subunit of photosynthetic bacteria *Rb. sphaeroides* and *Rb. capsulata* (Gennis *et al.*, 1993). Relevant to the present discussion is the observation that in a mutant where Asp252 was replaced by asparagine, cytochrome *b*-562 became, in flash kinetics experiments, rapidly and fully reduced and no reoxidation occurred. Conversely the quinol oxidation pathway was not influenced by this mutation (Gennis *et al.*, 1993). It is possible that a depressed protonation of this aspartate, as occurring in the presence of the steady-state ΔpH (alkaline shift of the N-phase pH), will result in decoupling of proton pump without significant depression of electron flow.

ACKNOWLEDGMENT

This work was supported by grant No. 95.02916.CT14 of CNR, Italy.

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