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Phospholipid Vesicles Containing Bovine Heart Mitochondrial Cytochrome *c* Oxidase Exhibit Proton Translocating Activity in the Presence of Gramicidin[1]

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Phospholipid vesicles containing bovine heart mitochondrial cytochrome *c* oxidase (COV) were characterized for electron transfer and proton translocating activities in the presence of the mobile potassium ionophore, valinomycin, and the channel-forming ionophore, gramicidin, in order to determine if the ionophores modify the functional properties of the enzyme. In agreement with previous work, incubation of COV with valinomycin resulted in a perturbation of the absorbance spectrum of oxidized heme aa_3 in the Soret region (430 nm); gramicidin had no effect on the heme aa_3 absorbance spectrum. Different concentrations of the two ionophores were required for maximum respiratory control ratios in COV; 40- to 70-fold higher concentrations of valinomycin were required to completely uncouple electron transfer activity when compared to gramidicin. The proton translocating activity of COV incubated with each ionophore gave a similar apparent proton translocated to electron transferred stoichiometry (H^+/e^- ratio) of 0.66 ± 0.10. However, COV treated with low concentrations of gramicidin (0.14 mg/g phospholipid) exhibited 1.5- to 2.5-fold higher rates of alkalinization of the extravesicular media after the initial proton translocation reaction than did COV treated with valinomycin, suggesting that gramicidin allows more rapid equilibration of protons across the phospholipid bilayer during the proton translocation assay. Moreover, at higher concentrations of gramicidin (1.4 mg/g phospholipid), the observed H^+/e^- ratio decreased to 0.280 ± 0.020, while the rate of alkalinization increased an additional 2-fold, suggesting that at higher concentrations, gramicidin acts as a proton ionophore. These results support the hypothesis that cytochrome *c* oxidase is a redox-linked proton pump that operates at similar efficiencies in the presence of either ionophore. Low concentrations of gramicidin dissipate the membrane potential in COV most likely by a channel mechanism that is different from the carrier mechanism of valinomycin, yet does not make the phospholipid bilayer freely permeable to protons.

Cytochrome *c* oxidase (COX[4] EC 1.9.3.1), the terminal electron carrier in the mitochondrial respiratory chain, reduces molecular oxygen to water (1) and conserves the energy of its oxidation–reduction reactions by the vectorial translocation of protons across the mitochondrial inner membrane (2). One proton (per electron transferred by the enzyme) is translocated across the mitochondrial inner membrane (3, 4), while an additional proton is abstracted from the intravesicular space (mitochondrial matrix) to be used in the catalytic reduction of oxygen (5, 6).

The observation of proton translocating activity in COX when *in vivo* (in the mitochondrial inner membrane) or when reconstituted into phospholipid vesicles (COV) requires the presence of membrane potential dissipating agents such as valinomycin plus potassium (2, 3, 7–9). In

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[4] Abbreviations used: COX, cytochrome *c* oxidase; COV, phospholipid vesicles containing cytochrome c oxidase; COV (+TX), phospholipid vesicles containing cytochrome *c* oxidase preincubated in Triton X-100; H^+/e^-, ratio of vectorial protons translocated per electron transferred; RCR, respiratory control ratio; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; RCR_{val}, respiratory control ratio determined in the presence of valinomycin; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; TX-100, Triton X-100; RCR_{gram}, respiratory control ratio determined in the presence of gramicidin; PL, phospholipid.

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either case, vectorial proton translocation across the phospholipid bilayer and consumption of protons by the enzyme during the oxygen reduction reaction create a large electrical potential difference across the membrane (2, 10). Addition of valinomycin collapses the membrane potential by coordinating potassium ions and carrying these ions across the phospholipid bilayer by a mobile carrier mechanism (11). Under these conditions, COX both in the mitochondrial membrane and in COV exhibits a maximum proton translocated to electron transferred ratio (H^+/e^-) approaching 1.0 (6).

Recently, Steverding and Kadenbach (12, 13) have shown that valinomycin directly interacts with COX as evidenced by a perturbation of the oxidized absorbance spectrum at 430 nm and a modification of the steady-state kinetics of cytochrome *c* interaction with COX upon the addition of valinomycin. Similar modifications of COX properties were also observed with another mobile potassium ionophore, nonactin (13). The conclusions drawn from these data were that valinomycin binds directly to COX and that the modified form of the enzyme translocates protons with the observed H^+/e^- stoichiometry of 1. Other work has shown that the electron transfer activity of COX is inhibited at high valinomycin concentrations, supporting the idea that valinomycin interacts with the enzyme (14–16).

The recent data obtained with valinomycin indirectly suggest that the extensively studied proton translocating function of COX could be due to a modification of the enzyme's conformational or even biophysical properties in the presence of this ionophore. In order to clarify the proton translocating activity of the enzyme, we investigated the proton-pumping activity of COX in COV utilizing the channel-forming ionophore, gramicidin (17, 18). We chose gramicidin as an ionophore because of its ability to mimic valinomycin in energy-dependent cation uptake and swelling in mitochondria and as a general membrane potential dissipating agent in other energy-transducing membranes (11). In the presence of low concentrations of gramicidin, we observe H^+/e^- ratios similar to those of COV treated with valinomycin. The spectral perturbation observed with valinomycin is not observed with gramicidin, supporting the idea that COX is a oxidation–reduction-linked proton pump with the stoichiometry first described by Wikstrom and associates (2, 3).

MATERIALS AND METHODS

Enzyme preparation. COX (7.0–9.0 nmol heme *a*/mg protein) was isolated from submitochondrial particles prepared by sonication of bovine heart mitochondria (19) as described by Yonetani (20). COX concentration was determined by using two extinction coefficients: 16.5 mM^{-1} cm^{-1} for reduced heme *a* at $\Delta A_{605-630\ nm}$ (21) and 164 mM^{-1} cm^{-1} for reduced minus oxidized heme aa_3 at 442 nm (22). Horse heart cytochrome *c* concentration was determined at 550 nm using 20.5 mM^{-1} (reduced minus oxidized) as the extinction coefficient (23). Protein was determined as described by Lowry *et al.* (24).

Preparation of phospholipid vesicles. COX was incorporated into phospholipid vesicles by cholate dialysis at 4°C, using the dialysis regime of DiBiase and Prochaska (25). L-α-Phosphatidylcholine at 40 mg/ml final concentration was dispersed in 66 mM Na^+ cholate and 100 mM Hepes NaOH, pH 7.2, by sonication at 0°C. COX was preincubated in 3 mg TX-100/mg COX at 0°C for 30 min [See Wilson and Prochaska (16) for full details] prior to the addition to the phospholipid mixture. The final concentration of heme aa_3 in the phospholipid vesicles [COV (+TX)] was 2–3 μM. Ninety-six percent of COX in the phospholipid vesicles had the cytochrome *c* binding domain oriented toward the extravesicular medium as determined by the method of Nicholls *et al.* (26) using a Gilford 2600 spectrophotometer. In addition, 91% of the enzyme was incorporated into the phospholipid vesicles as determined by an ultracentrifugation assay (16).

Difference absorbance spectra of COV (+TX) in the presence of valinomycin or gramicidin. The effects of gramicidin or valinomycin on the visible absorbance spectrum of oxidized COX in COV (+TX) were determined on an SLM DW-2c spectrophotometer in the split-beam mode in 100 mM sucrose, 100 mM KCl, and 10 mM Hepes NaOH, pH 7.2. Equivalent amounts of COV (+TX) [0.61 μM heme aa_3] were added to each cuvette and a baseline spectrum taken. Either gramicidin or valinomycin in methanol (20 μM for each) was then added to the sample cuvette with an equivalent volume of methanol added to the reference cuvette. After a 10-min incubation period at 20°C, the difference spectrum was taken and then corrected for the baseline spectrum.

Respiratory control assays. All electron transfer and respiratory control assays were performed in a thermostated cell at 25°C using an oxygen electrode (Yellow Springs Instrument Co., Model 17372). COV (+TX) (20–30 pmol heme aa_3) were assayed for electron transfer activity in 18 mM ascorbate, 40 μM cytochrome *c*, and 50 mM KH_2PO_4/K_2HPO_4, pH 7.4.

RCR and RCR_{val} of COV (+TX) were determined by the sequential addition of the ionophores to the assay mixture as described previously (16). RCR is defined as the rate of electron transfer in the absence of any gradients across the phospholipid bilayer (in the presence of 5.6 μM valinomycin to dissipate the membrane potential and 5.6 μM CCCP to collapse any pH gradient) divided by the rate of electron transfer in the absence of the uncoupling ionophores. RCR_{val} is defined as the rate of electron transfer of the phospholipid vesicles in the presence of valinomycin (5.6 μM) and CCCP (5.6 μM) divided by the rate of electron transfer in the presence of valinomycin alone. Similarly, RCR_{gram} is the rate of electron transfer in the phospholipid vesicles in the presence of gramicidin (0.1 μM) and CCCP (5.6 μM) divided by the rate of electron transfer in the presence of gramicidin alone. All electron transfer rates in COV (+TX) were corrected for the autooxidation of cytochrome *c*. The RCR is expected to have a higher value (>10) due to complete uncoupling of all gradients across the phospholipid bilayer, whereas RCR_{val} and RCR_{gram} will have lower values (<5) due to the presence of a membrane potential dissipating ionophore during the initial part of the assay (see Ref. (16) for complete details).

Proton translocation assays. Proton translocating activity was assayed at 25°C using a Corning Model 12 pH meter equipped with a combination electrode and a recorder as described previously (27). Electron transfer in COV (+TX) was initiated by the addition of ferrocytochrome *c*.

Reagents. Valinomycin was purchased from Sigma Chemical Co. or Calbiochem. Gramicidin, horse heart cytochrome *c* (Type III for electron transfer assays, Type VI for proton translocation assays), CCCP, TX-100, and L-α-phosphatidylcholine (Type II-S) were purchased from Sigma Chemical Co. Gramicidin purity was assessed by its ultraviolet absorbance spectrum and also by thin layer chromatography on Kodak silica gel plates using chloroform/methanol (85/15; v/v) as the developing solvent (28). Cholic acid (Aldrich Chemical Co.) was recrystallized from ethanol. Hepes was obtained from U.S. Biochemical Corp. or Calbiochem.

RESULTS

The Effect of Ionophores on the Visible Absorbance Spectrum of COX in COV (+TX)

The substitution of an alternative ionophore for measuring cytochrome *c* oxidase proton translocating activity was investigated due to the possible modification of COX by the interaction of valinomycin with the enzyme (12, 13). The effects of gramicidin on COX electron transfer and proton translocating activities were tested for two reasons: (i) gramicidin is a channel-forming ionophore, whereas valinomycin is a cyclic ionophore that performs its function as a mobile potassium carrier. The utilization of a membrane-spanning protein channel such as gramicidin could minimize the interaction of the ionophore with COX; (ii) gramicidin mimics valinomycin in its ability to stimulate energy-linked cation uptake and swelling in isolated mitochondria (11). Gramicidin also dissipates the membrane potential in both chloroplasts (as indicated by the abolition of the light-induced 515-nm absorbance change that is thought to reflect the buildup of a membrane potential in chloroplasts) and also mitochondria, suggesting similar cation permeabilities induced by the two ionophores in isolated membranes [see Ref. (11) and Discussion].

The visible absorbance spectrum of COX in COV (+TX) was determined in the presence and absence of the two ionophores (Fig. 1). Figure 1A shows that the γ band of oxidized heme *a* exhibits a red shift from 422 to 430 nm in valinomycin-treated COV (+TX) similar to that reported by Steverding and Kadenbach (12). The absorbance change occurs at concentrations of valinomycin [15–50 mol/mol heme aa_3] that support proton translocation by the enzyme. In gramicidin-treated COV (+TX) (Fig. 1B), no spectral shift is observed at concentrations of gramicidin (0.5–50 mol/mol heme aa_3) that allow proton translocating activity, suggesting that gramicidin does not interact with COX in the same manner as valinomycin. A similar valinomycin-induced red shift in the oxidized difference spectrum is also observed in phospholipid vesicles containing subunit III-deficient COX (data not shown), suggesting that subunit III is not the subunit location of the putative valinomycin binding site on COX.

Electron Transfer Activity and Respiratory Control Ratios of COV (+TX) Treated with Different Ionophores

If gramicidin mimics valinomycin in its mechanism of interaction with COX, then the concentration dependencies of their effect on the electron transfer activity and respiratory control ratios of the enzyme in COV (+TX) as well as the general shape of the curves for the ionophores' effects on these activities should be similar. In Fig. 2, the effects of various concentrations of ionophores on the respiratory control ratio, the respiratory control ratio in the presence of valinomycin (Fig. 2A) or the respiratory control ratio in the presence of gramicidin (Fig. 2B), and completely uncoupled electron transfer activity of COV (+TX) are presented. All RCR parameters are defined under Materials and Methods. Our previous work has shown that the RCR_{val} value of a preparation correlates with the proton-pumping activity of COX in phospholipid vesicles as a linear relationship (16), whereas the RCR value does not show this linearity.

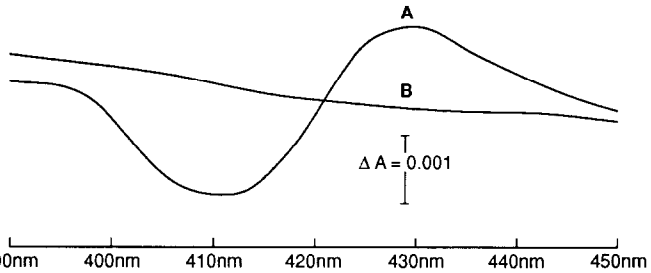

FIG. 1. The effects of gramicidin and valinomycin on the visible absorbance spectrum of COX in COV (+TX). The difference spectrum of heme aa_3 in COV (+TX) was determined in the presence of valinomycin (A) or gramicidin (B) as described under Materials and Methods using stoichiometries of 1.8 mg valinomycin/g PL or 3.1 mg gramicidin/g PL, respectively.

Figure 2A shows that the maximum uncoupled electron transfer activity was observed at 18 mg valinomycin/g phospholipid (PL) (5.3 μM valinomycin in the assay); however, at higher concentrations of valinomycin, significant inhibition of electron transfer activity was observed (40%). The maximum RCR value and the uncoupled electron transfer rate occur at a similar concentration of valinomycin, whereas the minimum RCR_{val} value occurs at higher concentrations of valinomycin (150 mg valinomycin/g PL). Figure 2B shows that the maximum electron transfer activity and RCR occur at the same concentration of gramicidin (0.71 mg gramicidin/g PL, 0.08 μM gramicidin in the assay), although the minimum RCR_{gram} value did not occur until much higher concentrations (5 mg gramicidin/g PL). Gramicidin caused a 20% inhibition of uncoupled electron transfer activity at the highest concentrations tested (12 μM gramicidin). Overall, the concentration dependencies for the effects of gramicidin and valinomycin on the electron transfer activity and RCR parameters were completely different. In fact, 40- to 70-fold less gramicidin was required to completely uncouple COX electron transfer activity, suggesting that the two ionophores may have different mechanisms of membrane potential dissipation. In mitochondria, gramicidin is 30-fold more efficient in dissipating the membrane potential than valinomycin (11).

The Effects of Gramicidin on Proton Translocating Activity of COX in COV (+TX)

Since the enzyme in COV (+TX) exhibited respiratory control in the presence of low concentrations of grami-

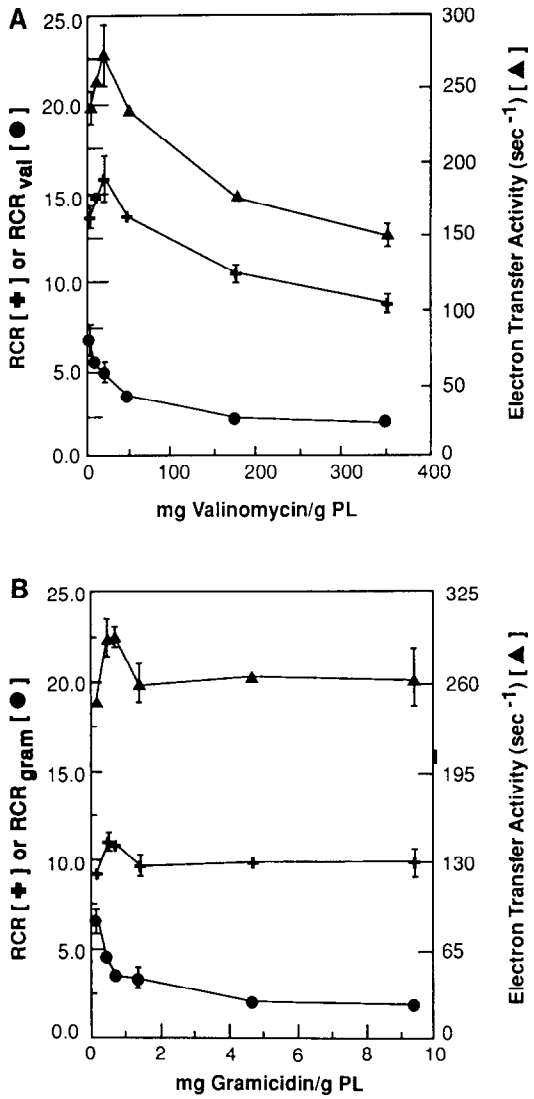

FIG. 2. The effect of various concentrations of ionophores on electron transfer activities and respiratory control ratios in COV (+TX). COV (+TX) were assayed for electron transfer activity in the presence and absence of ionophores using an oxygen electrode. The RCR, RCR_{val}, and RCR_{gram} were assessed from the electron transfer activity as described under Materials and Methods (16). (A) The RCR (+), RCR_{val} (●), and completely uncoupled electron transfer activity (▲) (assayed in the presence of valinomycin and CCCP) of COV (+TX) were determined in the presence of various concentrations of valinomycin (n = 3 for each point). (B) The RCR (+), RCR_{gram} (●), and the uncoupled electron transfer activity (▲) (in the presence of gramicidin and CCCP) were measured in the presence of various concentrations of gramicidin (n = 3 for each point).

cidin, the proton translocating activity of the enzyme in the presence of gramicidin was tested. Figure 3 shows proton translocation traces of COV (+TX) in the presence of valinomycin (A), low concentrations of gramicidin (B), and higher concentrations of gramicidin (C) at two COX turnovers (1 COX turnover = $4e^-$ for cytochrome c/heme aa_3). Upon the addition of ferrocytochrome c to a solution of COV (+TX) (Fig. 3), an acidification was followed by an alkalinization. The acid phase represents vectorial proton translocation, whereas the alkaline phase reflects consumption of protons in the oxygen reduction reaction in the intravesicular space (6). In the presence of valinomycin (Fig. 3A) or low concentrations of gramicidin (Fig. 3B), similar extents of acidification were observed; however, the time course for the alkaline phase of the proton translocation reaction in gramicidin-treated COV (+TX) was much faster. At higher concentrations of gramicidin (Fig. 3C, 10-fold higher than in Fig. 3B), the extent of acidification was decreased with an accompanying increase in the alkalinization rate. Similar data were obtained for COV (+TX) that had undergone five turnovers ($20e^-$/heme aa_3).

Apparent H^+/e^- ratios were calculated from the data by using the extrapolation method of Krab and Wikstrom (3) and corrected for substoichiometric extents of alkalinization in the presence of CCCP (Table I) (29). At the low gramicidin concentration (Fig. 3B) and in the presence of valinomycin (Fig. 3A) an H^+/e^- ratio of 0.64 was observed, while the H^+/e^- ratio at the higher concentration of gramicidin (Fig. 3C) decreased to 0.26. Table I shows that the observed H^+/e^- stoichiometry in the presence of valinomycin is similar to the H^+/e^- stoichiometry in the presence of low concentrations of gramicidin (at

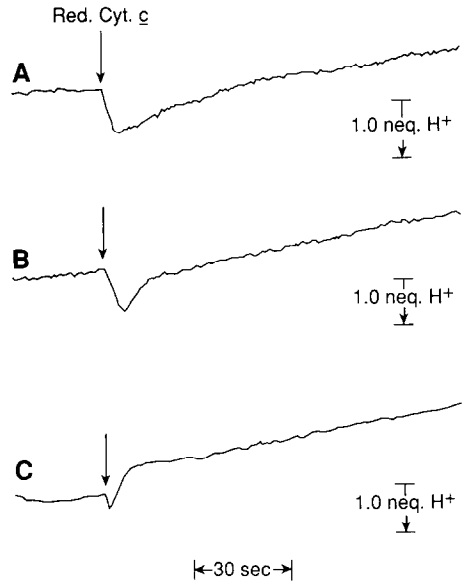

FIG. 3. Proton translocating activity of COV (+TX) in the presence of valinomycin or gramicidin. Approximately 1.6 nequivalent of ferrocytochrome c was added to 0.20 nmol (heme aa_3) COV (+TX) in 100 mM sucrose, 100 mM KCl, and 0.25 mM Hepes NaOH, pH 7.2, and changes in pH were monitored as described under Materials and Methods. In trace A, 2.8 mg valinomycin/g phospholipid was preincubated with COV (+TX), whereas in traces B and C, 0.14 and 1.41 mg gramicidin/g phospholipid were preincubated with COV (+TX). Apparent H^+/e^- ratios were calculated using the extrapolation method of Krab and Wikstrom (3). The apparent H^+/e^- ratios [corrected by the method of Casey (29)] at two COX turnovers (8 mol cytochrome c/mol aa_3) were (A) 0.63, (B) 0.65, and (C) 0.26.

TABLE I

The Effect of Different Ionophores on the Proton Translocating Activity and Alkalinization Rates in COV (+TX)

Ionophore[a]	Apparent H^+/e^- stoichiometry[b]		Alkalinization rate (NEQ OH^-/s)[c]	
	Two turnovers	Five turnovers	Two turnovers	Five turnovers
Valinomycin	0.66 ± 0.10	0.43 ± 0.05	0.035 ± 0.004	0.068 ± 0.010
Gramicidin	0.63 ± 0.04	0.38 ± 0.04	0.088 ± 0.008	0.112 ± 0.008

[a] COV (+TX) were prepared as described under Materials and Methods at approximately 2 μM heme aa_3. The assays were performed with 2.8 mg valinomycin/g phospholipid and 0.14 mg gramicidin/g phospholipid, respectively.

[b] Apparent H^+/e^- stoichiometries were calculated as described under Materials and Methods (2). One enzyme turnover is defined as four cytochrome c molecules oxidized/heme aa_3. The numbers of determinations at two and five enzyme turnovers were 18 and 16 for valinomycin and 12 and 5 for gramicidin, respectively.

[c] Rates of alkalinization were estimated from proton translocation traces in Fig. 2 and treated as pseudo-first-order processes. The numbers of determinations were 13 and 8 for valinomycin and 6 and 3 for gramicidin at two and five enzyme turnovers, respectively.

both two or five enzyme turnovers), emphasizing that similar data for both ionophores were obtained at two different concentrations of cytochrome c added.

Correlation of the Rates of Alkalinization in the Presence of Gramicidin in the Proton Translocation Assay with the H^+/e^- Ratio

Faster rates of alkalinization in the proton translocation assay (Fig. 3) are hypothesized to be due to an increase in the endogenous proton permeability of the COV and such preparations yield correspondingly lower H^+/e^- ratios (although this is not always the case, see Ref. (16)). Table I shows that the rates of alkalinization for valinomycin-treated COV (+TX) are from 1.5- to 2.5-fold slower than those for gramicidin-treated COV (+TX), yet the apparent H^+/e^- ratio is similar for both treatments. Gramicidin stimulates the alkalinization at both two and five enzyme turnovers, but the proton-pumping activity is similar to that of valinomycin-treated COV (+TX). In these measurements, a faster alkalinization rate does not correlate with a decreased H^+/e^- ratio. These data support our earlier observations that the rate of alkalinization in these assays is not due exclusively to changes in the proton permeability of the membrane (16).

However, the rates of alkalinization within the concentration dependence of gramicidin alone correlate with the apparent H^+/e^- ratio (Fig. 4). At 0.14 mg gramicidin/g PL, an optimum apparent H^+/e^- ratio was observed. At higher concentrations of gramicidin, a decrease in the apparent H^+/e^- ratio was observed with a corresponding increase in the alkalinization rate. If the concentration was increased to 5 μM gramicidin, no proton translocating activity was observed (A. J. Lincoln and L. J. Prochaska, unpublished observations). Only a rapid alkalinization was observed which is indicative of complete uncoupling or membrane leakiness to protons. At the lowest concentration of gramicidin tested (0.047 mg gramicidin/g PL), the apparent H^+/e^- ratio and the alkalinization rate were both low, suggesting that the amount of gramicidin added was insufficient to dissipate the membrane potential [in agreement with previously published work (3)].

DISCUSSION

Proton translocation by COX both in the mitochondrial inner membrane and when reconstituted into phospholipid vesicles was first shown by Wikstrom and associates (2, 3) to require the addition of a membrane potential-dissipating ionophore such as valinomycin. Measurements of the membrane potential formed in COV suggest that a gradient of 66 mV is formed upon the initiation of electron transfer (10, 30). Dissipation of the membrane potential is required for measurement of proton translocating activity in COV because the small size of COV (diameter of 250 Å, Ref. (31)) causes a buildup of the membrane potential that inhibits COX electron transfer activity (30). Valinomycin has been used to dissipate the membrane potential in COV due to valinomycin's selectivity for potassium over other monovalent ions and also its ability to carry ions across a phospholipid bilayer by the mobile carrier mechanism.

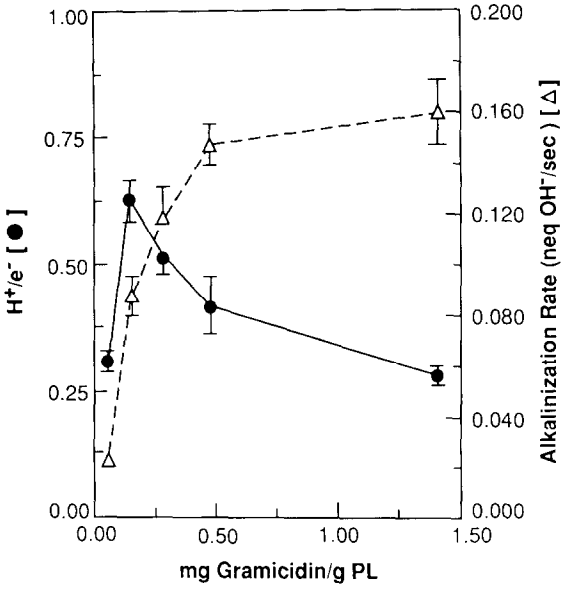

FIG. 4. The effect of various concentrations of gramicidin upon the apparent H^+/e^- ratio and the alkalinization rate in COV (+TX). COV (+TX) were assayed for proton translocating activity at various concentrations of gramicidin. The apparent H^+/e^- ratios (●) and the corresponding alkalinization rates (Δ) were determined at two enzyme turnovers. These data represent three individual preparations of COV (+TX) and the number of determinations was from 4 to 12 for all gramicidin concentrations tested.

The recent evidence that valinomycin interacts with COX suggests that the proton-pumping measurements performed on COV and the interpretations drawn from these experiments may have been clouded by a potential artifact. In this work, we tested COX proton translocating activity utilizing an alternative ionophore, gramicidin, in order to clarify the results obtained with valinomycin. We used gramicidin because of its channel-forming mechanism of action, thereby limiting the interaction of the ionophore with COX (17, 18). Also, gramicidin has a similarity in its physiological action in mitochondria with valinomycin (11).

Our results show that COX proton translocating activity occurs in the presence of low concentrations of gramicidin and the data obtained qualitatively mimic those obtained with valinomycin; COX pumps protons with an apparent H^+/e^- ratio of approximately one. Gramicidin does not share the same mechanism of interaction with COX as valinomycin as evidenced by the lack of a change in the enzyme's visible absorbance spectrum upon the addition of gramicidin. Although the apparent H^+/e^- ratio is the same for both ionophores, the rate of alkalinization in the proton-pumping assay (Fig. 3) is faster for gramicidin than for valinomycin, suggesting that the mechanism of dissipation of the membrane potential by the two ionophores is different. Additional evidence for different mechanisms of action is that the concentration dependencies for the maximum stimulation of electron transfer activity and the respiratory control activities by the two ionophores are different (Fig. 2). Interestingly, the addition of high concentrations of gramicidin (5 μM) leads to complete abolition of proton translocating activity with an accompanying increase in the rate of alkalinization, suggesting that at high concentrations, gramicidin acts as proton ionophore (see below).

The mechanism of the dissipation of the membrane potential in COV by gramicidin is unclear. The ion selectivity of gramicidin channels is $H^+ > NH_4^+ > Cs^+ > Rb^+ > K^+ > Na^+ > Li^+$ as determined at 0.1 M concentrations using black lipid membranes (32, 33). Using the ion permeabilities derived from these experiments, protons are from 20- to 50-fold more permeable than potassium ions in the gramicidin channel. In liposomes, Deamer (34) estimated that the gramicidin channel is 100-fold more permeable to protons than to potassium ions and when unit permeability coefficients were calculated, protons were found to be 50,000 more permeable than potassium ions. It is unlikely that protons are dissipating the membrane potential in our experiments because we observe similar values of the apparent H^+/e^- ratio in COV treated with either gramicidin or valinomycin. In addition, the proton translocating activity of COV is not observed in the presence of proton-specific ionophores, such as CCCP (35). However, it is more likely that potassium ions not protons are dissipating the membrane potential due to the 1×10^6 molar excess of potassium (0.1 M) over protons (10^{-7} M) under the conditions of our proton translocation assay. Therefore, it is highly unlikely that gramicidin (at low concentrations) is acting as an uncoupler in our experiments.

One additional experiment supports gramicidin acting as a membrane potential dissipating agent in our experiments. The electron transfer rate of COV (+TX) in the presence of high concentrations of gramicidin is not stimulated by the addition of valinomycin, suggesting that gramicidin is acting both as a proton ionophore and as a membrane potential dissipating ionophore under these experimental conditions. If a similar experiment is performed with an ionophore specific for protons only such as CCCP, subsequent addition of valinomycin is required for maximum electron transfer activity.

Proton translocating activity is observed when low concentrations of gramicidin are added to COV (+TX) (Fig. 3). Under these conditions, there are from 0.5–2.5 gramicidin monomers per phospholipid vesicle (250 Å diameter, based on intravesicular volume measurements). Thus, the number of gramicidin molecules per COV approximates the required dimeric species for the ion-conducting channel form of gramicidin (18). In our experiments, gramicidin is added only from the extravesicular medium, suggesting that the antiparallel dimers of gramicidin which exist in aqueous solution partition into the phospholipid bilayer and reorient into the active, β-helical, dimeric form of gramicidin (17). Also, recent evidence shows that the conformation and oligomeric state of gramicidin are dependent on the carrier solvent of the ionophore (36). In our experiments, the solvent is methanol and gramicidin is a monomer, which can easily undergo a conformational change into the active, ion translocating β-helical conformer (36). Other recent data suggest that addition of gramicidin from one side of a bilayer results in some of the gramicidin monomers forming ion-conducting channels (37, 38). In any event, the previous work with mitochondria suggests that effects of gramicidin occur by addition of the ionophore from only the external media (11), supporting our results in COV.

An alternative explanation of our proton translocation data obtained with gramicidin is that gramicidin binds to COX and alters its functional activity in a manner similar to valinomycin, but does not cause a perturbation of the heme aa_3 spectrum of the enzyme. While our experiments cannot completely exclude this explanation, we think it is unlikely for the following reasons: (i) 40- to 70-fold less gramicidin is required to stimulate electron transfer and proton translocation than valinomycin. Proton translocating activity is observed at 0.5–2.5 gramicidin monomers/COV, suggesting that the dimeric ion translocating unit of gramicidin is required for membrane potential dissipation in our experiments. If gramicidin does indeed bind to COX, the binding site (for a gramicidin dimer) or sites (two binding sites, one for each monomer) would have a much higher affinity than the putative valino-

mycin-binding site on COX due to their vastly different concentration dependencies on electron transfer and proton translocating activities of the enzyme. We have been unable to observe any significant effects of gramicidin on the heme aa_3 absorbance spectrum or the maximum rate of electron transfer even at the highest gramicidin/PL ratios (20% or less inhibition of electron transfer), suggesting that the previously described dimeric gramicidin channel is most likely the functioning species in our experiments (18); (ii) Nonactin and valinomycin require the presence of K^+ ions to cause the spectral perturbation in heme aa_3 in the enzyme, whereas ionophores such as nigericin and 18-crown-6 ether with K^+ have no effect on the absorbance spectrum of the enzyme, suggesting that not all ionophores have the ability to bind to COX (12). In our view, our results are best explained by gramicidin acting as a membrane potential dissipating peptide channel.

Our conclusion is that cytochrome *c* oxidase is a redox-associated proton pump with a previously determined apparent H^+/e^- ratio of one. The interaction of valinomycin with COX does not dramatically affect the maximum proton translocating activity of the enzyme. Our work shows that gramicidin (at low concentrations) can effectively substitute for valinomycin as a membrane potential dissipating agent. Gramicidin apparently does not interact with COX in a manner similar to valinomycin, thus avoiding any possible perturbations of COX function similar to those induced by valinomycin. Our data do not address the possible effects of valinomycin on the electron transfer reactions of the enzyme or the valinomycin-induced perturbation of the regulation of COX conformations as described previously (12, 13).

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REFERENCES

1. Capaldi, R. A. (1990) *Annu. Rev. Biochem.* **59,** 569–596.
2. Wikstrom, M. K. F., and Saari, H. T. (1977) *Biochim. Biophys. Acta* **462,** 347–361.
3. Krab, K., and Wikstrom, M. (1978) *Biochim. Biophys. Acta* **504,** 200–214.
4. Casey, R. P., and Azzi, A. (1983) *FEBS Lett.* **154,** 237–242.
5. Wikstrom, M. (1984) *Nature (London)* **308,** 558–560.
6. Thelen, M., O'Shea, P. S., Petrone, G., and Azzi, A. (1985) *J. Biol. Chem.* **260,** 3626–3631.
7. Proteau, G., Wrigglesworth, J. M., and Nicholls, P. (1983) *Biochem. J.* **210,** 199–205.
8. Nicholls, P., and Shaughnessy, S. (1985) *Biochem. J.* **228,** 201–210.
9. Moroney, P., Scholes, T. A., and Hinkle, P. C. (1984) *Biochemistry* **23,** 4991–4997.
10. Singh, A. P., and Nicholls, P. (1986) *Arch. Biochem. Biophys.* **245,** 436–445.
11. Pressman, B. C. (1976) *Annu. Rev. Biochem.* **45,** 501–531.
12. Steverding, D., and Kadenbach, B. (1990) *Biochem. Biophys. Res. Commun.* **160,** 1132–1139.
13. Steverding, D., and Kadenbach, B. (1990) *J. Bioenerg. Biomembr.* **22,** 197–205.
14. Gregory, L. C., and Ferguson-Miller, S. (1989) *Biochemistry* **28,** 2655–2662.
15. Nicholls, P., Cooper, C. E., and Kjarsgaard, J. (1988) *in* Advances in Membrane Biochemistry and Bioenergetics (Kim, C. H., Tedeschi, H., Diwan, J. J., Salerno, J. C., Eds.), pp 311–321, A. R. Liss, New York.
16. Wilson, K. S., and Prochaska, L. J. (1990) *Arch. Biochem. Biophys.* **282,** 413–420.
17. Andersen, O. S. (1984) *Annu. Rev. Physiol.* **46,** 531–548.
18. Wallace, B. A. (1990) *Annu. Rev. Biophys. Chem.* **19,** 127–157.
19. Azzone, G. F., Colonna, R., and Ziche, B. (1979) *in* Methods in Enzymology (Fleischer, S., and Packer, L., Eds.), Vol. 55, pp. 46–50, Academic Press, New York.
20. Yonetani, T. (1967) *in* Methods in Enzymology (Estabrook, R. W., and Pullman, M. E., Eds.), Vol. 10, pp. 332–336, Academic Press, New York.
21. Briggs, M. M., and Capaldi, R. A. (1977) *Biochemistry* **16,** 73–77.
22. Van Gelder, B. F. (1966) *Biochim. Biophys. Acta* **118,** 36–48.
23. Margoliash, E., and Frohwirt, N. (1959) *Biochem. J.* **71,** 570–577.
24. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275.
25. DiBiase, V. A., and Prochaska, L. J. (1985) *Arch. Biochem. Biophys.* **243,** 668–677.
26. Nicholls, P., Hildebrandt, V., and Wrigglesworth, J. M. (1980) *Arch. Biochem. Biophys.* **204,** 533–543.
27. Prochaska, L. J., and Reynolds, K. A. (1986) *Biochemistry* **25,** 781–787.
28. Bauer, K., Roskoski, R., Kleinkauf, H., and Lipmann, F. (1972) *Biochemistry* **11,** 3266–3271.
29. Casey, R. P. (1986) *in* Methods in Enzymology (Fleischer, S., and Fleischer, B., Eds.), Vol. 126, pp. 13–21, Academic Press, New York.
30. Wrigglesworth, J. M., Cooper, C. C., Sharpe, M. A., and Nicholls, P. (1990) *Biochem. J.* **270,** 109–118.
31. Mueller, M., and Azzi, A. (1985) *J. Bioenerg. Biomembr.* **17,** 385–393.
32. Hladky, S. B., and Haydon, D. A. (1972) *Biochim. Biophys. Acta* **274,** 294–312.
33. Myers, V. B., and Haydon, D. A. (1972) *Biochim. Biophys. Acta* **274,** 312–322.
34. Deamer, D. W. (1987) *J. Bioenerg. Biomembr.* **19,** 457–479.
35. Casey, R. P., Chappell, J. B., and Azzi, A. (1979) *Biochem. J.* **182,** 149–156.
36. Bano, M. C., Braco, L., and Abad, C. (1991) *Biochemistry* **30,** 886–894.
37. O'Connell, A. M., Koeppe, R. E., and Andersen, O. S. (1990) *Science* **250,** 1256–1259.
38. Clement, N. R., and Gould, J. M. (1981) *Biochemistry* **20,** 1544–1548.