STUDIES ON THE CHARACTERISTICS OF A PROTON PUMP IN PHOSPHOLIPID VESICLES INLAYED WITH PURIFIED COMPLEX III FROM BEEF HEART MITOCHONDRIA

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1. Introduction

It is well documented that the respiratory chain functions as a proton pump [1]. Two views have been put forward to describe the details of this pumping action. Mitchell's original hypothesis described the pump as a vectorial transmembrane movement of the hydrogen and electron carriers [1]. More recently, Papa et al. [2–4] proposed that the redox-proton pump is generated by a linkage between the oxido-reduction of the metal centers of electron carriers and the protonic equilibrium of acidic groups of the apoprotein. Consistent with this suggestion is the demonstration of a proton carrier in the Complex III region of the respiratory chain [3–5]. In this region, no classical hydrogen carriers are known [6–8]. More recently, the presence of a proton pump has been demonstrated in phospholipid liposomes containing purified Complex III [9]. In the present paper we report a study on some properties of this proton pump, demonstrating its electrogenic nature and pH dependency.

2. Methods and materials

Complex III was prepared by the methods of Rieske et al. [6]. The redox and spectral properties of this preparation have been described [10]. Liposomes were prepared from soybean phospholipids and then inlayed with Complex III as described by Hinkle and Leung [9]. In all experiments final dialysis of the Complex III vesicles was made against 150 mM KCl [9].

The reaction media for proton release and cytochrome *c* reduction measurements contained: 150 mM KCl, 5 mM $MgCl_2$, 15 μM cytochrome *c*, 0.28 mg Complex III protein/ml, and 1 mM KCN when added. Routinely, 3 ml of this solution was prepared, the pH was adjusted to near pH 7.5 with KOH, and the samples were divided equally for use in proton release and cytochrome *c* reduction experiments. The solutions were allowed to equilibrate in a closed vessel maintained at 25°C for 2 min and electron transfer was routinely initiated by addition of 40 μM duroquinol (DQH_2).

Changes in pH were measured using a Radiometer, combination pH electrode (GK 2320C) connected to a Radiometer, model 26, pH meter (Model 26). Response time for the apparatus was determined to be approx. 0.3 sec.

DQH was prepared by reducing a 20 mM methanolic solution of duroquinone (DQ) with a few grains of borohydrid, followed by acidification of the reduced solution to between pH 6.5 and 7 with 0.1 M HCl [11]. Under these conditions DQH_2 remained reduced for several hours. Addition of small aliquots (4 μl) of DQH_2 to suspensions of phospholipid vesicles did not alter the pH of the media because of the buffering power of the vesicles, and did not, therefore, interfere with measurements of initial proton release.

Cytochrome *c* was measured at 550 *minus* 540 nm using a mM extinction of 21 [12]. Protein was measured by the biuret procedure (B).

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3 Results

Fig.1A shows the tracings for H^+ release and cytochrome *c* reduction by Complex III vesicles under a flux of argon. About 3–4 min after addition of 40 μM DQH_2 (final concentration) the released protons were again taken up and a concomitant oxidation of cytochrome *c* was observed. Similar results were observed with only Complex III and can be attributed to small amounts of contaminating cytochrome oxidase. Although cytochrome aa_3 could not be detected spectrally, the above reactions were inhibited by azide and KCN, but not by iron chelating agents, TTFA and bathophenanthroline. In addition, complete oxidation of DQH_2 was dependent upon catalytic amounts of cytochrome *c*. This slow reoxidation of DQH_2 (5% of that in particles [14]) could significantly influence the steady state of H^+ release when such low concentrations of substrate are used. For this reason it is important to assure that cytochrome oxidase activity is inhibited, if accurate determinations of proton translocation are to be expected.

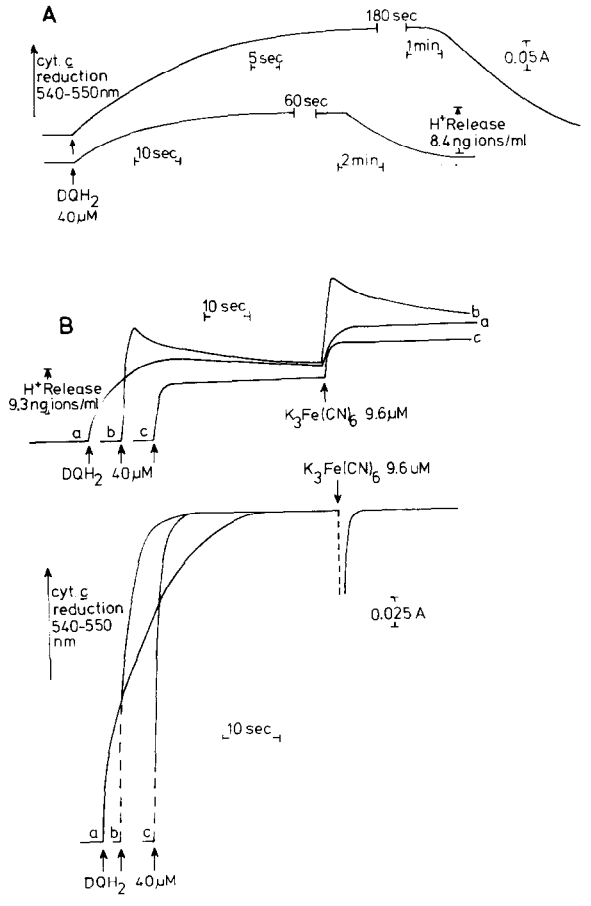

Fig.1. Proton release and cytochrome *c* reduction in liposomes containing Complex III. Conditions are described in Methods. The final volume was 1.5 ml, the pH was 7.5 and the temperature was 25°C. A) The reaction was carried out in a closed vessel under a flux of argon (< 1 ppm oxygen); B) 1 mM KCN was present. Trace a, control, no additions; trace b, 0.1 μg/ml of valinomycin; and trace c, 0.1 μg/ml valinomycin plus 0.1 μg/ml nigericin.

Fig.1B shows a typical experiment carried out in the presence of KCN. Under these conditions the rates of H^+ release and cytochrome *c* reduction were increased, and both the steady state reduction levels of cytochrome *c* and the pH gradient were stabilized. The $\rightarrow H^+/e$ ratio could thus be accurately measured. The $\rightarrow H^+/e$ ratio can also be determined after a subsequent addition of ferricyanide pulses to DQH_2-reduced Complex III vesicles (fig.1B), enabling us to compare the two pulse methods. In both types of experiments antimycin inhibited cytochrome *c* reduction and H^+ release. FCCP could also replace nigericin in abolishing the pH gradient established in the presence of valinomycin.

The $\rightarrow H^+/e$ ratios obtained in several experiments are summarized in table 1. Complex III, in the absence of phospholipid vesicles gives a $\rightarrow H^+/e^-$ ratio slightly less than, but very near, to 1.0. This is expected from the oxidation of DQH_2. Furthermore, in these preparations the $\rightarrow H^+/e^-$ ratio obtained after oxidation of DQH_2 is unaltered by valinomycin or valinomycin + nigericin. In contrast, the $\rightarrow H^+/e^-$ ratios obtained in Complex III vesicles oxidizing DQH_2 were greater than 1.0 in the control. This ratio was further increased in the presence of valinomycin, and lowered to near 1.0 in the presence of valinomycin + nigericin. These data indicate the presence of a proton pump in Complex III vesicles. Similar indications were also obtained using ferricyanide pulses (table 1).

Fig.1B also shows that valinomycin decreases the t 1/2 for H^+ release and cytochrome *c* reduction in Complex III vesicles. No effect of valinomycin was observed on Complex III alone (not shown). These data indicate that electron transport and H^+ release are controlled in the vesicles by the membrane potential. In all the cases, the t 1/2 in the presence of

Table 1
Comparison between the H^+/e^- ratios obtained with pulses of $K_3FE(CN)_6$ and DQH_2 in purified Complex III and liposomes containing Complex III

Additions		Complex III		Liposomes of Complex III
	n	H^+ released/cyto *c* reduced	n	H^+released/cyto *c* reduced
None	4	0.79 ± 0.03	10	1.43 ± 0.10
Valinomycin	4	0.68 ± 0.01	10	1.75 ± 0.11
Valinomycin + nigericin	4	0.70 ± 0.02	10	1.10 ± 0.07
	n	H^+ released/$K_3Fe(CN)_6$ added	n	H^+ released/$K_3Fe(CN)_6$ added
None	4	0.85 ± 0.03	10	0.98 ± 0.06
Valinomycin	4	0.74 ± 0.05	10	1.50 ± 0.05
Valinomycin + nigericin	4	0.77 ± 0.11	10	0.85 ± 0.05

Experimental conditions as in Methods and fig.1b. The values obtained with Complex III and Complex III vesicles were obtained using 4 different preparations of Complex III. n = number of experiments. The values are expressed as the mean ± S.E.

nigericin and valinomycin is too small (> 1 sec) to be accurately determined with our apparatus.

Fig.2A is a typical experiment showing the effects of pH on the extent of H^+ release, cytochrome *c* reduction and the $\rightarrow H^+/e^-$ ratio after a pulse of DQH_2. The $\rightarrow H^+/e^-$ ratio increased sharply above pH 7.0. This is due only to an increased release of protons. The extent of cytochrome *c* reduction is unaffected by increasing pH. The data were corrected for antimycin

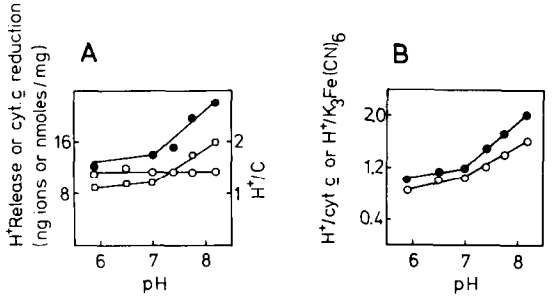

Fig.2. Effect of pH on proton release and cytochrome *c* reduction in liposomes containing Complex III. Conditions are as described in fig.1 with the exception that 1 mM KCN and 0.1 μg/ml valinomycin were present. A) A typical experiment showing: (●—●) proton release; (○-○) cytochrome *c* reduction; (□-□) $\rightarrow H^+/e^-$ ratio in liposomes of Complex III during reductant pulses of DQH_2; B) Mean values summarized for 8 experiments with 3 different preparations of Complex III, showing $\rightarrow H^+/e^-$ ratios obtained with DQH_2 (●-●) or ferricyanide (○-○) pulses.

sensitive H^+ release and therefore, represent electron transport-dependent pH changes. The rates of both H^+ release and cytochrome *c* reduction are also increased with increasing pH. The t 1/2 values observed above pH 7.4 were too low to be accurately determined under the conditions of our assay. However, below pH 7.4 the t 1/2 for proton release is estimated to be half of that for cytochrome *c* reduction (not shown).

The effects of pH on the $\rightarrow H^+/e^-$ ratio are summarized for 8 experiments in fig.2B. Ratios obtained using either DQH_2 or ferricyanide pulses are compared. The data demonstrate an apparent pK_a of near 7.0 for initiation of proton pump activity. The slightly lower $\rightarrow H^+/e^-$ ratio routinely obtained using ferricyanide pulses apparently result from either an underestimation of H^+ release due to a prior generation of a H^+ gradient during equilibration with DQH_2, or to an overestimation of the amount of ferricyanide undergoing reduction.

4. Discussion

The data reported in table 1, showing a $\rightarrow H^+/e^-$ ratio greater than 1.0, as expected from only the oxidation of DQH_2, confirms the presence of a proton pump which takes up protons from the inside of Complex III vesicles and releases them outside (see

also [9]). The increase in H^+ release caused by valinomycin indicates that the pump is a transmembrane process of electrogenic nature. The electrogenic nature of the proton pump was suggested by Hinkle and Leung [9] but was not demonstrated experimentally. The presence of a proton pump in purified Complex III negates the original belief [1] that the H^+ carrier in this region of the respiratory chain is a classical hydrogen carrier. The components of Complex III are well described [6–8] and do not include appreciable concentrations of such hydrogen carriers.

The proton pump in Complex III vesicles has an apparent pK of approx. 7.0, above which proton pump action is initiated. The opposite is observed in submitochondrial particles, where increasing pH decreased the H^+/O ratio [15]. This latter pH dependency was explained as resulting from an underestimation of the ΔpH due to compensatory movements of permeant weak acids [15]. In Complex III vesicles, however, this does not appear to be an important consideration since neither the media nor the vesicles contain components which could act in this manner. A second explanation for differences in the pH dependency of the proton pumps in Complex III vesicles and submitochondrial particles [15] might be related to the orientation of Complex III in phospho lipid vesicles. With cytochrome c as the electron acceptor, only the proton pump in mitochondrially-oriented particles (cytochrome c_1 on the outside) will be measured. Thus, an increase in proton pump activity in Complex III vesicles at alkali pH should be related to the pK of an ionizable group on the *outside* of the membrane. In contrast, the decrease in proton pump activity measured in submitochondrial particles at alkaline pH [15] would reflect the ionization of a group, or groups, on the *inside* of the membrane. This would be consistent with the idea that the proton pump is controlled by the pK_a of two groups on different sides of the membrane, and that low pH inside (carrier protonated) and high pH outside (carrier deprotonated) promotes proton transport out. In this case the pH does not need to represent that of the bulk phases, but rather only the H^+ concentration near the protonated components involved. This view suggests that proton translocation would occur down a concentration gradient, at least under conditions of high external pH.

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