# EFFECTS OF $K^+$ DIFFUSION POTENTIALS ON DUROQUINOL-CYTOCHROME *c* REDUCTASE ACTIVITY CATALYZED BY COMPLEX III INCORPORATED INTO LIPOSOMES

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## 1. Introduction

It has been shown that beef heart quinol-cytochrome *c* reductase (complex III) incorporated into phospholipid vesicles can drive proton translocation [1,2]. In such vesicles, quinol-cytochrome *c* reductase activity is inhibited in the absence of uncoupling agents or valinomycin + $K^+$ [1–3], and it is generally assumed that inhibition is due to the presence of a membrane potential or an electrochemical gradient of protons developed between the internal and external osmotic spaces [4–6]. To date, however, little information has been published showing that quinol-cytochrome *c* reductase can be influenced in reconstituted vesicles by ionic movements between the two osmotic spaces. In the present study we show that duroquinol-cytochrome *c* reductase activity can be brought into equilibrium with a $K^+$ diffusion potential, and that the enzyme is inactivated as the potential becomes more negative inside the vesicles. In addition, we also show, using the cationic dye safranine, that vesicles containing complex III can drive the formation of a membrane potential.

## 2. Methods

Complex III was prepared from beef heart mitochondria as in [8]. Phospholipid vesicles containing complex III were prepared from soybean phospholipids (Sigma) as in [1,2] using the cholate dialysis procedure [9]. The phospholipids were extracted with acetone [9] prior to use. Vesicles were prepared in 50 mM Tris–$SO_4$, pH 7.5, containing various concentrations of KCl or NaCl as indicated in the figure legends. Safranine uptake and duroquinol-cytochrome *c* reductase activity [2] were routinely measured in 1 ml buffer containing 50 mM Tris–$SO_4$, pH 7.5, 1 mM EDTA, and varying amounts of KCl or NaCl to maintain osmolarity with the vesicles. Reduction of cytochrome *c* was measured at 550–540 nm and safranine uptake was measured at 530–578 nm [10].

Membrane potentials were calculated from the initial concentrations of $K^+$ inside, $[K^+]_{in}$, and outside, $[K^+]_{out}$, using the Nerst equation:

$$E_m = 59 \log \frac{[K^+]_{in}}{[K^+]_{out}}$$

For these calculations, $[K^+]_{in}$ was assumed to be the same as the buffer $[K^+]$ during preparation of the vesicles. The concentration ratio of safranine, $[S^+]_{in}/[S^+]_{out}$, was calculated assuming that a decrease in absorbance at 530–578 nm is proportional to safranine uptake, $[S^+]_{in}$, and that 100% uptake was achieved with no external $K^+$ present.

## 3. Results

Figure 1 shows a typical experiment using liposomes containing complex III. As reported [11,13], a valinomycin-induced $K^+$ diffusion potential drives the

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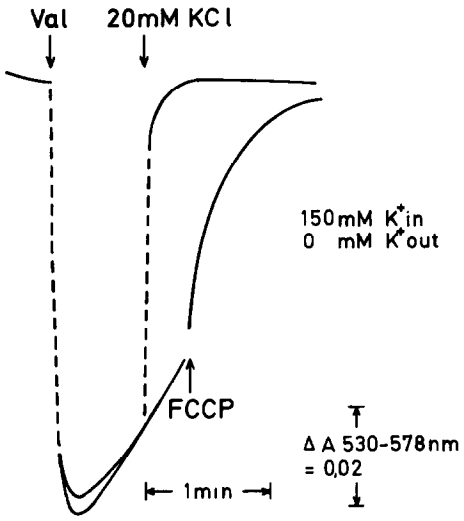


Fig.1. $K^+$-driven uptake of safranine into phospholipid vesicles reconstituted with beef heart complex III. Vesicles were prepared in 50 mM Tris–$SO_4$, pH 7.5, 150 mM KCl and 1 mM EDTA. The reaction media contained 50 mM Tris–$SO_4$, pH 7.5, 150 mM NaCl, 1 mM EDTA, 75 μg phospholipid and 18 μM safranine in total vol. 1 ml. Valinomycin and FCCP were 0.5 μg/ml and 1 μM respectively.

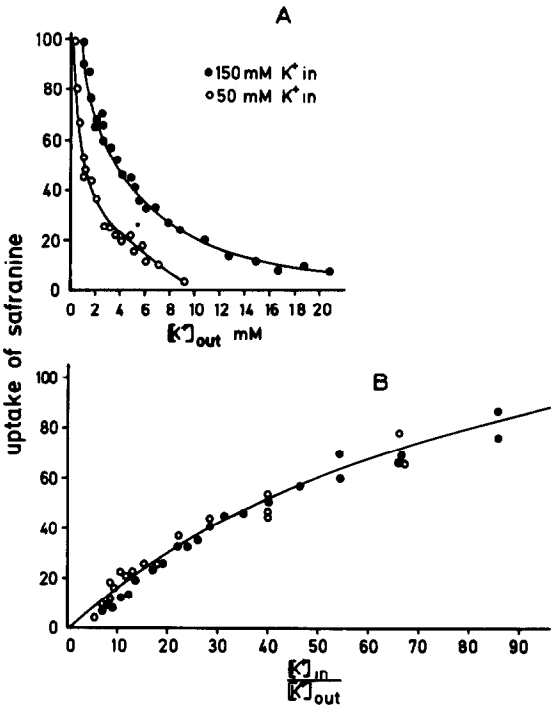


Fig.2. Effects of internal $K^+$ concentration on safranine uptake by complex III-phospholipid vesicles. Experimental conditions were as given in fig.1 with the exception that vesicles were made in the presence of either 50 mM or 150 mM KCl. As the external KCl concentration was raised, the external NaCl concentration was decreased proportionally to maintain osmolarity with the internal KCl.

uptake of safranine. Safranine uptake can be prevented or reversed by any reagent which collapses the $K^+$ gradient (fig.1). That safranine probes an electrical event across the liposomal membrane is shown in fig.2. As expected, the absolute external $[K^+]$ needed to prevent safranine uptake is dependent upon the $[K^+]$ inside the vesicles (fig.2A). However, when safranine uptake is plotted against the $K^+$ concentration gradient (fig.2B), all points fall on the same line, regardless of absolute internal $[K^+]$. A plot of the log of the accumulation ratio of safranine, log $[S^+]_{in}/[S^+]_{out}$, against the membrane potential, gives a straight line with a slope of 1 (data not shown). It should be noted, however, that the absolute absorbance changes obtained for a particular liposomal preparation are strongly dependent upon the ratio of safranine to phospholipid, and must be taken into consideration when comparing quantitative results obtained with different liposomal preparations. Optimal absorbance changes are obtained with a ratio of 0.25 μmol safranine/mg phospholipid.

Figure 3A shows that duroquinol-cytochrome *c* reductase activity is controlled by a $K^+$ diffusion potential. Since the membrane potential is calculated from the $K^+$ concentration gradient it is expressed as a positive value. It is clear, however, that as the membrane potential becomes more negative, as measured by safranine uptake, the enzyme become inactivated (fig.3A). Safranine uptake and enzyme inactivation appear to be in equilibrium with each other (fig.3B) and with the $K^+$ diffusion gradient (fig.3C).

Duroquinol-cytochrome *c* reductase also drives the formation of a membrane potential in liposomes, as indicated by the uptake of safranine (fig.4). Safranine uptake can be prevented or abolished by FCCP or valinomycin + $K^+$, conditions which also activate duroquinol-cytochrome *c* reductase [1–3]. The slight net increase in absorbance observed in the presence of FCCP or valinomycin is due to interference from reduced cytochrome *c* at these wavelengths.

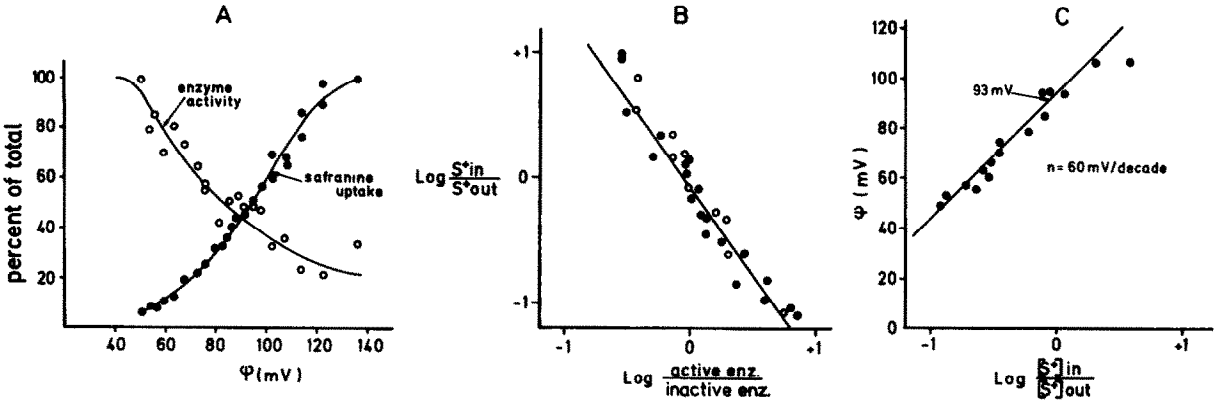


Fig.3. Control of duroquinol-cytochrome *c* reductase in complex III-phospholipid vesicles by $K^+$ diffusion potentials. Conditions were as in fig.2 with the exception that 60 μM duroquinol and 12 μM cytochrome *c* were present in the media during the measurements of enzyme activity. Duroquinol-cytochrome *c* reductase activity and safranine uptake were initiated by the addition of 0.5 μg valinomycin. The enzymatic rates shown in the figure are the initial rates obtained after addition of valinomycin.

## 4. Discussion

The present study shows that:

1. Duroquinol-cytochrome *c* reductase in reconstituted phospholipid vesicles can be brought into equilibrium with a $K^+$ diffusion potential, and becomes inactivated as the membrane potential becomes more negative inside the vesicle.
2. Duroquinol-cytochrome *c* reductase can drive the formation of a membrane potential in complex III vesicles, as shown by the uptake of safranine.
3. The diffusion potential-driven uptake of safranine by complex III vesicles follows the Nernst equation and is a reliable qualitative proble of transmembrane potentials.

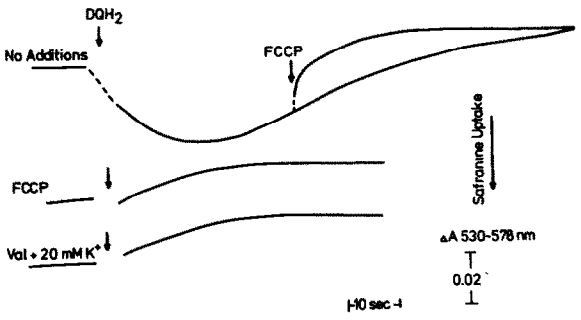


Fig.4. Formation of a membrane potential driven by duroquinol-cytochrome *c* reductase incorporated into phospholipid vesicles. The conditions of the assay were as in fig.1 and fig.3.

Complex III vesicles do not catalyze the reduction of cytochrome *c* unless an uncoupling agent or valinomycin + $K^+$ is present [1–3]. This has been interpreted to mean that the first turnovers of the enzyme create a redox-driven electrochemical proton gradient which is sufficient to inhibit electron-transfer [1–3]. The present observation that duroquinol-cytochrome *c* reductase is able to drive the uptake of safranine shows that the enzyme can form a membrane potential, and is in keeping with the above interpretation. The present findings also clearly show that both safranine uptake and duroquinol-cytochrome *c* reductase activity can be brought into equilibrium with a $K^+$ diffusion gradient (fig.3), indicating that the enzyme can be controlled by ionic movements between the internal and external osmotic spaces. The effect of the ΔpH (pH gradient) on duroquinol-cytochrome *c* reductase activity has not yet been successfully determined due:

(i) To the lack of reliable methods for measuring internal pH in liposomal systems where the proton movement is directed outwards.

(ii) To the fact that isolated duroquinol-cytochrome *c* reductase is strongly pH dependent [3], making acid–base transition experiments [13] difficult to interpret.

It should be mentioned, however, that in several

experiments we have observed valinomycin-activated rates to be 20–40% lower than FCCP-activated rates. This difference in enzyme activity can be due to the influence of the ΔpH which is enhanced in the valinomycin-treated vesicles.

We have attempted to use the $K^+$-driven uptake of safranine as a standard to quantitate [11–13] membrane potentials formed under conditions of electron transport inhibition [1–3]. The results show that the duroquinol-cytochrome *c* reductase is half-maximally activated by a $K^+$ diffusion potential of 90–95 mV. In contrast, duroquinol-cytochrome *c* reductase drives the formation of a potential no greater than 10 mV, as measured by safranine (data not shown), even under conditions where electron transport is controlled [1–3]. This discrepancy is most easily explained if, like cytochrome oxidase [14,15], complex III is incorporated into only a small percent of the vesicles. In this case, all vesicles would accumulate safranine in the presence of a $K^+$ diffusion potential, whereas only a small percentage would accumulate safranine when driven by duroquinol-cytochrome *c* reductase. Quantitation of the membrane potential based upon $K^+$-driven safranine uptake [12,13] would, thus, lead to a considerable underestimation of the enzyme-driven membrane potential.

It would thus appear that the membrane potentials calculated from $K^+$ diffusion gradients are probably nearest to those which actually control duroquinol-cytochrome *c* reductase activity (fig.3). Even these values are open to question, however, since they are based on the assumption that the $K^+$-driven safranine response is independent of the size of the liposomal vesicle. The size of the vesicle prepared by cholate dialysis can vary considerably [14]. It now appears that proteins can be inserted into vesicles of a restricted and uniform size [14]. Quantitation of membrane potentials in liposomal vesicles using safranine as a probe is, thus, associated with problems apparently not experienced with intact rat liver mitochondria [12,13].

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