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ENERGIZED TRANSPORT OF POTASSIUM IONS IN THE ABSENCE OF VALINOMYCIN BY CYTOCHROME *c* OXIDASE-RECONSTITUTED VESICLES

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Valinomycin-independent energized uptake of K^+ was observed in cytochrome *c* oxidase reconstituted proteoliposome. The rate of K^+ influx was proportoinal to the magnitude of electron flux. The energized uptake of K^+ was abolished by *p*-trifluoromethoxycarbonylcyanide phenylhydrazone or by nigericin. Using the safranine fluorescence technique, it was demonstrated that even in the absence of valinomycin, liposomes and proteoliposomes reconstituted with cytochrome *c* oxidase are able to discriminate between Na^+ and K^+ and show a preference for K^+ in the presence of excess Na^+.

Introduction

Reconstituted phospholipid vesicles containing cytochrome *c* oxidase have been shown to generate a membrane potential [1,2] and ΔpH [3] when ascorbate in the presence of cytochrome *c* and TMPD is used as electron donor (for a recent review, see Ref. 4). In the presence of valinomycin, respiration-dependent accumulation of K^+ has been also demonstrated by cytochrome oxidase vesicles [5–11]. In later studies [8–11], the ratio K^+/e^- was found to be up to 2.0. In addition to cytochrome oxidase vesicles, lysophosphatidylcholine-dispersed cytochrome oxidase preparations have been reported [12–13] to catalyze energy-dependent K^+ uptake during an oxygen pulse, even in the absence of valinomycin. The K^+/e^- ratio estimated in this kind of experiment was found to be close to 1.0. Accumulation of certain monovalent and divalent cations by cytochrome oxidase vesicles, in the absence of valinomycin has been reported previously [14,15]. However, at the high salt concentrations normally used (10 mM or more), it is difficult to determine which ions are taken up and which are released by cytochrome oxidase vesicles under such conditions. Indeed, the continuous alkalinization of the proteoliposomal interior in the absence of ionophore demands an appropriate co- or countertransport of ions [3]. It is generally understood that model membranes, including liposomes, are not freely permeable to K^+ in the absence of valinomycin (see, e.g., Ref. 16); and in contrast to biological membranes, they exhibit a low permeability to ions in general, and an apparent lack of discrimination between Na^+ and K^+ (reviewed in Ref. 17). However, Fry and Green [14] have shown that proteoliposomes reconstituted with a subfraction of cytochrome oxidase, in particular subunit I (M_r 40000), have increased passive permeability to monovalent and divalent cations, like Na^+, K^+, Rb^+, and Ca^{2+}.

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Abbreviations: TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; FCCP, *p*-trifluoromethoxycarbonylcyanide phenylhydrazone; Hepes, 4-(2-hydroxyethyl)piperazineethanesulphonic acid.

In the absence of a Ca^{2+} ionophore, energized uptake of Ca^{2+} by cytochrome oxidase vesicles has also been demonstrated [18]. On the basis of these results, it was suggested by Fry and Green [14,15] that cytochrome *c* oxidase acts as a nonspecific ion-channel, upon its incorporation into liposomes.

In this study, we report the energized uptake of K^+ by cytochrome oxidase vesicles in the absence of K^+ ionophore. In addition, it is also shown here, that liposomes- and cytochrome *c* oxidase-reconstituted proteoliposomes exhibit differential passive permeabilities to Na^+ and K^+.

Materials and Methods

Cytochrome *c* oxidase was prepared from bovine heart by the method of Kuboyama et al. [19], with Tween 80 substituting for Emasol, and stored at −75°C as described before [20]. The final preparation of the enzyme had a haem/protein ratio of 8.5 μmol/g and a maximal turnover (electrons/cytochrome aa_3) of 435 S^{-1} at 30°C in the presence of 5 mg/ml phosphatidylcholine (asolectin, Type IVS, Sigma), 0.5% Tween 80, 67 mM sodium phosphate (pH 7.4), with ascorbate and cytochrome *c* as substrates.

Reconstitution of cytochrome oxidase vesicles was performed as outlined by Proteau et al. [21], except that in place of potassium phosphate, either 50 mM sodium phosphate or 50 mM Hepes-NaOH buffers (pH 7.4) were used. All liposomes and aa_3-reconstituted proteoliposomes were prepared using soybean phosphatidylcholine Type IVS (Sigma).

The passive permeabilities of liposomes and cytochrome oxidase vesicles to Na^+ and to K^+ were determined by loading the vesicles with either Na^+ or K^+ and diluting them into Na^+- and K^+-free media, respectively, in the presence of safranine. The efflux of Na^+ or K^+ from the interior of the vesicles, down their concentration gradients, into the outside medium, led to the generation of a membrane potential (internally negative). The subsequent movement of the positively charged dye, safranine, into the vesicle interior was followed fluorometrically. The fluorescence of safranine was excited at 525 nm and the emission measured by a photomultiplier placed at 90° to the cuvette using a Oriel long pass filter (No. 5131, 50%, 575 nm) and the multipurpose cuvette system described by Kraayenhof et al. [22].

In passive permeability experiments, the 2.8 ml reaction mixture contained either 50 mM sodium phosphate or 50 mM potassium phosphate buffers (pH 7.4), 5 μM safranine and liposomes or proteoliposomes containing 0.18 mg/ml of phospholipid. In the case of energized quenching of safranine fluorescence, the 2.8 ml reaction mixture contained 50 mM Hepes-NaOH buffer (pH 7.4), 14.3 mM sodium ascorbate, 9 μM cytochrome *c*, 5 μM safranine and cytochrome oxidase vesicles containing 0.274 μM cytochrome *c* oxidase and 3.6 mg/ml phospholipid. The residual concentration of Tween 80 (as a contaminant from cytochrome oxidase) was no more than 0.0001%.

Potassium uptake was monitored in a thermostatically controlled glass-chamber fitted with a magnetic stirrer, using a K^+-sensitive electrode (Radiometer, model No. F2312K) and a calomel reference electrode, with a double salt bridge (K701) connected to a Radiometer digital pH meter (PHM 64) and recorder (Rec 67, Servograph, Radiometer, Copenhagen, Denmark). The 7.8 ml reaction mixture contained either 50 mM sodium phosphate or 50 mM Hepes-NaOH buffers (pH 7.4), 6.4 mM sodium ascorbate, 190 μM TMPD, 6.4 μM cytochrome *c*, 30–40 μM KCl and proteoliposomes containing 0.14–0.54 μM cytochrome *c* oxidase and 3.2 mg/ml phospholipid. The final concentration of Tween 80 (as a contaminant from the oxidase) was between 0.0018 and 0.0004%. No attempt was made to remove contaminating potassium present in the reactants, including buffers, phospholipids, cytochrome *c* and cytochrome *c* oxidase. The total concentration of contaminating potassium in the above reaction mixture was found to be between 240 and 250 μM. The final concentration of K^+ as KCl in a typical experiment was between 280 and 300 μM. In every experiment, the K^+ electrode was first set to zero chart deflection units in the presence of buffers and proteoliposomes. Then, it was calibrated by successive additions of known concentrations of KCl and when a stable base-line was reached, K^+ uptake was energized by addition of ascorbate, TMPD and cytochrome *c*.

Cytochrome *c* (type VI, horse heart), sodium

ascorbate, TMPD (dihydrochloride) and valinomycin were Sigma products. Nigericin was obtained from Calbiochem-Behring, La Jolla, CA. FCCP was a gift of Dr. P.G. Heytler of Dupont.

Results and Discussion

In the presence of oxidizable substrate and cytochrome *c*, cytochrome oxidase will generate an internally negative membrane potential in cytochrome oxidase vesicles [1,2,5,23,24]. K^+, a monovalent cation, could be accumulated by cytochrome oxidase vesicles in response to this membrane potential, given an appropriate transport system conferring K^+ permeability on the cytochrome oxidase vesicle membrane. As can be seen from Fig. 1, a spontaneous respiration-dependent uptake of K^+ was consistently observed using cytochrome *c* oxidase-containing proteoliposomes when ascorbate in the presence of TMPD and cytochrome *c* was used as the electron donor under aerobic conditions. In the absence of either cytochrome *c* or ascorbate, or both, no uptake of K^+ occurred (Fig. 1). Similar results were obtained with liposomes lacking cytochrome oxidase. When electron flux was controlled either by omitting the TMPD from the reaction mixture or by varying the amount of oxidase, the rate and extent of K^+ uptake by cytochrome oxidase vesicles was found to be proportional to the magnitude of electron flux (Figs. 1 and 2). Similar results were also obtained when electron flux was controlled by the use of cytochrome *c* oxidase inhibitors, cyanide and azide (results not shown). Upon initiation of ferrocytochrome *c* oxidation, K^+ apparently moves into the cytochrome oxidase vesicles until an equilibrium or steady-state condition is established; upon anaerobiosis, K^+ flows out of the vesicle restoring the initial level of K^+ in the external medium. Thus, a K^+ concentration gradient is created upon energization in the absence of valinomycin and eliminated during de-energization. Analogous results were also obtained in the presence of valinomycin (Fig. 3, compare traces a and b). However, in the presence of this iono-

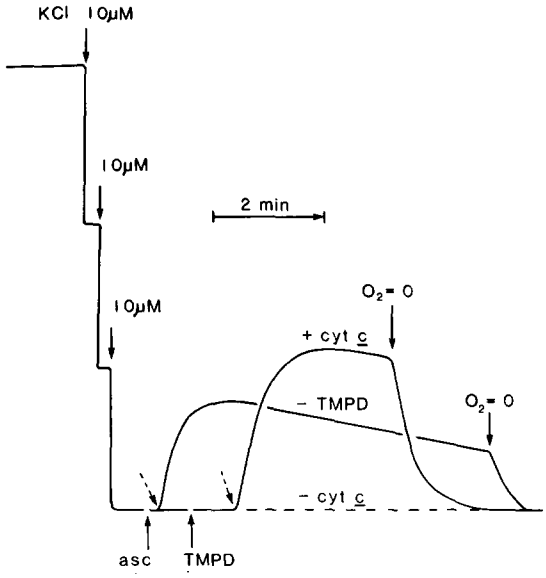

Fig. 1. Kinetics of energized uptake of potassium by cytochrome *c* oxidase-reconstituted proteoliposomes in the presence and absence of cytochrome *c* and TMPD. The 7.8 ml reaction mixture contained 50 mM Hepes-NaOH buffer (pH 7.4), 6.4 mM cytochrome *c*, 190 μM TMPD, 6.4 mM sodium ascorbate and proteoliposomes containing 0.28 μM cytochrome *c* oxidase and 3.2 mg/ml of phospholipid. At the dashed arrows, cytochrome *c* was added.

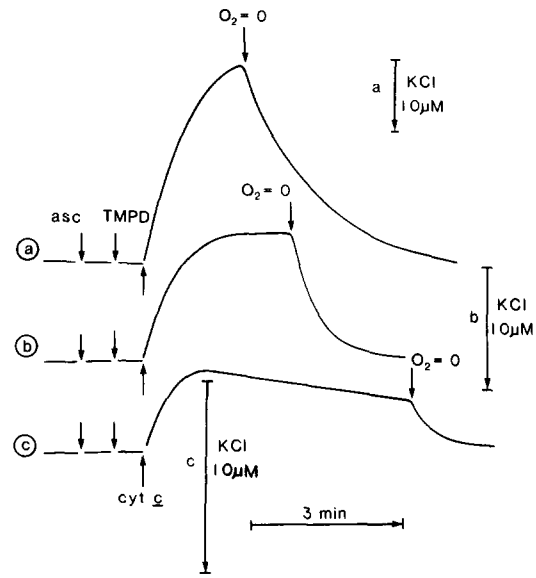

Fig. 2. Effect of different concentrations of cytochrome *c* oxidase on the kinetics of energized uptake of K^+ by proteoliposomes. The final concentrations of cytochrome *c* oxidase in traces, a, b and c were 0.536, 0.268 and 0.134 μM, respectively. The concentrations of the other components of the reaction mixture were the same as given in the legend to Fig. 1.

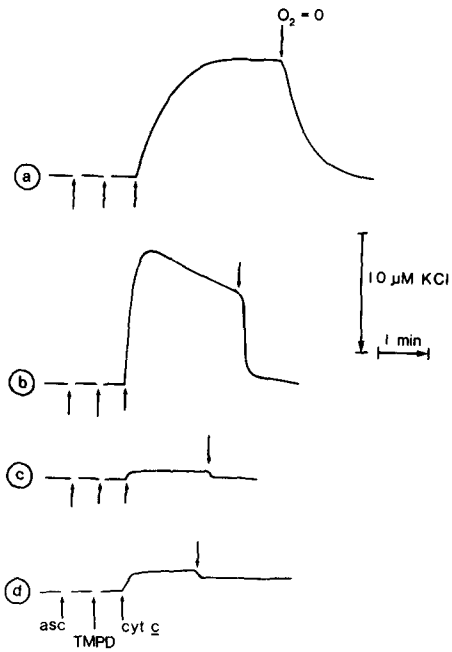

Fig. 3. Kinetics of energized uptake of potassium by cytochrome oxidase vesicles in the absence (trace a) and presence of 100 ng/ml valinomycin (trace b), and its inhibition by 100 ng/ml nigericin (trace c) or 82 ng/ml FCCP (trace d). The concentrations of the other components of the reaction mixture were the same as given in the legend to Fig. 1.

phore, the initial rate of K^+ uptake was at least 5-times faster than in its absence. For example, in the presence of valinomycin, the steady-state level of K^+ uptake was reached within 22 s, while the same process took 102 seconds in its absence. In both cases, the eventual steady-state level of K^+ accumulation was the same.

In the absence of valinomycin, the steady-state value of K^+ uptake is maintained until the onset of anaerobiosis; in its presence, the steady-state K^+ level was maintained for only a few seconds. K^+ then began to efflux at a slow rate till anaerobiosis was reached. This slow efflux of K^+ prior to anaerobiosis, which occurs only in the presence of valinomycin, may be due to an exchange of K^+ for Na^+ catalyzed by this ionophore at the very high Na^+ concentrations used relative to K^+. Parallel experiments show no synchronous decline of internal alkalinity, and the total internal cation level must therefore be maintained.

The valinomycin-independent uptake of K^+ was inhibited 80–90% by nigericin (Fig. 3, trace c). For example, in the absence of nigericin, an appreciable K^+ gradient was generated by cytochrome oxidase vesicles upon energization, which was reduced to negligible levels in the presence of nigericin. The remaining K^+ gradient was completely abolished upon anaerobiosis (Fig. 3, trace c). In the presence of protonophore, FCCP (Fig. 3, trace d), the K^+ gradient was completely abolished. During respiration, both H^+ gradients and K^+ gradients are generated across the vesicular membrane [5–11]. When the H^+ gradient is abolished by FCCP, the K^+ uptake is simultaneously abolished. Although, in the presence of nigericin, the membrane potential is maintained, both H^+ and K^+ concentration gradients are quickly equilibrated, and thus no net accumulation of K^+ is observed.

The nigericin and FCCP effects are consistent with the observation that respiring proteoliposomes become internally alkalinized by 0.3–0.4 pH units both in presence and absence of valinomycin, as measured either by vesicle-entrapped phenol red [3,25] or pyranine [26]. This OH^- gradient, which requires a concomitant counterion gradient, is also abolished by FCCP or nigericin.

It is evident from the above results that there is a specific mediator of K^+ permeability in cytochrome oxidase vesicles (even in the presence of high levels of Na^+) which allows K^+ uptake under appropriate energization conditions. An attempt was made to determine the relative passive permeabilities towards Na^+ and K^+ of liposomes and aa_3-reconstituted proteoliposomes prepared from the same soybean lipids, by observation of the fluorescence of a membrane-potential-sensitive dye [27].

The fluorescence of the positively charged dye, safranine, when added to the external medium in the presence of liposomes, is quenched when liposomes are made internally negative by the efflux of a cation down its concentration gradient [28]. In the absence of ion gradients, no quenching of safranine by liposomes occurred (Fig. 4, dashed line). A differential quenching of safranine fluorescence was however observed when liposomes were preloaded either with Na^+ or K^+ and diluted into Na^+- or K^+-free media, respectively (Fig. 4, traces

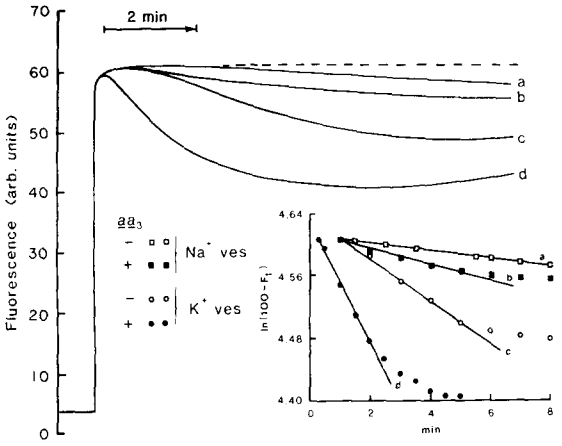

Fig. 4. Correlation between relative passive permeability of liposomes (trace a and c) and cytochrome *c* oxidase-containing proteoliposomes (trace b and d) towards Na^+ (traces a and b) and K^+ (traces c and d) as determined by safranine fluorescence. In order to estimate the initial rate of fluorescence decay caused by the efflux of either K^+ or Na^+, a logarithmic replot of the fluorescence data was made and shown as the inset. For such a logarithmic plot, the fluorescence level reached within 30 s after the addition of dye was normalized to 100 arbitrary units in all cases (traces a–d), and the fraction of fluorescence quenched with time *t*, (F_t), due to cation efflux was subtracted from 100. The natural log of ($100 - F_t$) was then plotted against *t*. From the initial slopes of these traces, the rate of safranine fluorescence quenching and hence the approximate rates of efflux of Na^+ and K^+ from liposomes and proteoliposomes can be estimated.

a and c). Under similar conditions, the quenching of safranine was enhanced further differentially in Na^+- and K^+-loaded proteoliposomes reconstituted with cytochrome *c* oxidase (Fig. 4, traces b and d). A replot of the fluorescence data is shown in Fig. 4 (inset), from which estimates of the relative passive permeabilities of Na^+ and K^+ can be made for liposomes and proteoliposomes.

In contrast to the findings of Fry and Green [14,15], in the presence of cytochrome *c* oxidase, the relative passive permeability of Na^+ in proteoliposomes is increased 2-fold, whereas the K^+ permeability is increased 4.4-fold (Fig. 4, inset traces b and d). The passive permeability of liposomes, made in the absence of cytochrome *c* oxidase, was 2.2-fold greater for K^+ than for Na^+ (Fig. 4, inset, traces a and c). Analogous results were obtained by other investigators [29] for liposomes made from egg phosphatidylcholine. However, the ability of 'pure' phospholipid vesicles to discriminate between univalent cations is also dependent on the kind of phospholipid present [30]. It was pointed out in earlier studies [30–32] that phosphatidylcholine, phosphatidylethanolamine, and phosphatidic acid vesicles do not show any such specificity with regards to permeabilities to K^+, Rb^+ and Na^+. The differences between this and other studies [14,30–32] may be due to methodological differences. For example, it was pointed out in a recent study [27] on ion permeabilities of rabbit intestinal brush-border membrane vesicles that tracer techniques (used in earlier studies) provide estimates of the absolute magnitudes of permeability coefficients, while fluorescence measurements of potential sensitive dyes yield estimates of both relative and absolute ion permeabilities. This may explain why no differences were observed in the passive permeabilities of either asolectin or egg phosphatidylcholine vesicles including aa_3-reconstituted proteoliposomes to K^+ and Na^+ in the studies of Fry and Green [14,15].

In contrast to the findings of Young et al. [33] in liposomes prepared from synthetic saturated phospholipids, Tween 80 even at a concentration of 0.0007% had no effect on the passive permeability of the present vesicles to Na^+ and K^+.

Differential permeability of cytochrome oxidase vesicles for K^+ and Na^+ has been also demonstrated under energized conditions. Fig. 5 shows that the fluorescence of safranine in the presence of cytochrome oxidase vesicles was quenched by 40–50% when ascorbate was used as electron donor in the presence of cytochrome *c*. The steady-state level of fluorescence was maintained for about 1 min (Fig. 5, traces a, b, c and d) and then started to decline, prior to anaerobiosis, at a rate dependent on the concentration of K^+ and the dye in the external medium [2]. No such effect was observed in the absence of K^+ (Fig. 5, trace e). Upon anaerobiosis, the fluorescence abruptly increased due to safranine efflux from cytochrome oxidase vesicles into external medium, in every case. An oxidase-driven uptake of K^+, but not of Na^+, is evidently responsible for the slow increase of fluorescence (efflux of safranine) during the steady state. Although, in Fig. 5 the external K^+ concentration was 15–40 mM, while the internal level was only 0.2 mM, similar results were also ob-

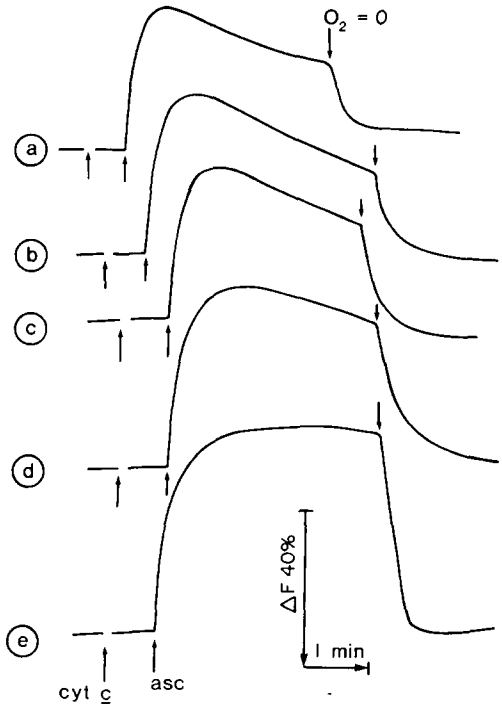

Fig. 5. Effect of potassium salts on the steady-state fluorescence intensity of safranine in the presence of cytochrome *c* oxidase-reconstituted proteoliposomes. The 2.8 ml reaction mixture contained 50 mM Hepes-NaOH buffer (pH 7.4), 14.3 mM sodium ascorbate (except in trace d, where 14.3 mM potassium ascorbate was used), 9 μM cytochrome *c*, 5 μM safranine, and proteoliposomes containing 0.274 μM cytochrome *c* oxidase and 3.58 mg/ml of phospholipid. In addition to 50 mM Hepes-NaOH buffer (pH 7.4), the reaction mixture also contained 40 mM KCl (trace a), 40 mM K_2SO_4 (trace b), 40 mM KNO_3 (trace c), 14.5 mM K^+-ascorbate (trace d) or 40 mM NaCl (trace e).

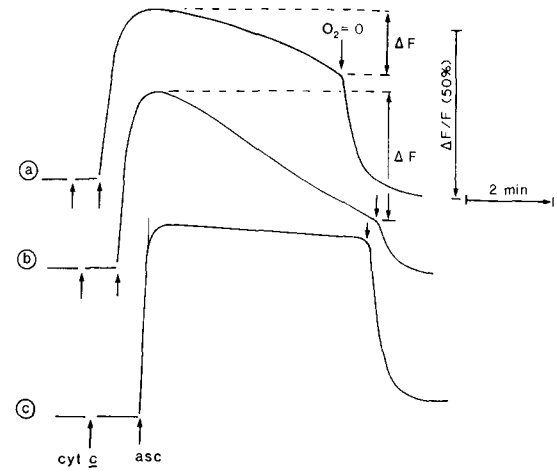

Fig. 6. Effect of K^+ gradients and nigericin on the steady-state fluorescence intensity of safranine in the presence of cytochrome *c* oxidase-reconstituted proteoliposomes. In traces a, b and c, the concentratoin of K^+ inside of cytochrome oxidase vesicles was the same (90 mM), whereas on the outside of the cytochrome oxidase vesicles, it was 6.5 mM (trace a) and 90 mM (traces b and c). Nigericin was present at a final concentration of 0.7 μg/ml in trace c. The other conditions were the same as described in the legend to Fig. 5.

tained when the K^+ gradient was reversed (Fig. 6). After the steady state is reached, the decay of safranine fluorescence (safranine efflux) was very slow when cytochrome oxidase vesicles contained high K^+ levels inside (90 mM) and low K^+ levels outside (6.5 mM) (Fig. 6, trace a), and faster when the K^+ concentrations on both sides of cytochrome oxidase vesicles were the same (90 mM on each side; cf. Fig. 6, trace b). In trace a, in order to expel safranine from the inside to the outside of cytochrome oxidase vesicles, K^+ has to move against its own concentration gradient; in trace b, it moves with the concentration gradient. So the rate of K^+ movement which depends upon the activity of cytochrome *c* oxidase, may be a critical factor in the efflux of safranine. No safranine efflux was observed in the presence of nigericin, even in K^+-containing media (Fig. 6, trace c), which suggests that K^+ influx into cytochrome oxidase vesicles is dependent on H^+ efflux out of oxidase vesicles. In comparison to controls without nigericin, in the presence of this ionophore, the rate of oxygen uptake (derived from the time to reach anaerobiosis) was increased both in a low K^+ (Fig. 3, cf. traces a and c) and in a high K^+ (Fig. 6, cf. traces b and c) medium. The small stimulatory effect of nigericin on oxygen uptake in the latter medium may be due to a slower rate of respiration caused by the absence of TMPD in this system.

The rate of K^+-dependent safranine efflux cannot be quantitatively equated with the rate of net K^+ uptake as observed with a K^+ electrode (Fig. 1), since the process is dependent upon the concentration of dye [2]. At low dye concentrations (1 μM), all the dye is tightly bound to the inner surface of cytochrome oxidase vesicles and very little is free to be exchanged with K^+. Under the above conditions, even in K^+-containing media,

the fluorescence increase of safranine after steady state is achieved, is very slow. Therefore, a critical dye concentration may be required to demonstrate dye-K^+ exchange under energized conditions by cytochrome oxidase vesicles. We conclude that, in contrast to the findings of Fry and Green [13–15], and even in the presence of a 50-fold excess of Na^+ over K^+ in the external medium, the oxidase is able to drive active uptake of K^+ in preference to Na^+. From Fig. 5 (traces a, b, c and d) it is also evident that the oxidase-driven entry of K^+ does not depend on the anion (e.g., Cl^-, NO_3^-, SO_4^{2-}, ascorbate) present in the incubation medium. Thus, even in the absence of valinomycin, cytochrome *c* oxidase has the capability to energize the preferential transport of K^+ over Na^+ into proteoliposomes.

Acknowledgements

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