Effects of detergents and cytochrome *c* binding on scalar and vectorial proton ejection by proteoliposomes containing cytochrome oxidase

P. NICHOLLS* and S. SHAUGHNESSY†

Department of Biological Sciences, Brock University, St. Catharines, Ontario L2S 3A1, Canada

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1. The detergent lauryl maltoside abolishes respiratory control and proton ejection by cytochrome *c* oxidase-containing proteoliposomes over a narrow concentration range. Expression of cryptic activity (inward-facing oxidase) is released over the same concentration range. Catalytic functions ($V_{\max}$ and K_m) of the enzyme are not changed by the detergent. Lipid micelles containing detergent bind approximately the same amount of cytochrome *c* as do vesicles containing an equivalent amount of lipid. 2. Uncoupler-insensitive proton release is seen when proteoliposomes are pulsed with ferrocytochrome *c* at low ionic strength. Such uncoupler-insensitive acidification is not seen at higher ionic strength, nor with oxygen pulses of anaerobic solutions previously incubated with cytochrome *c*. Vesicles at low ionic strength catalyse cytochrome *c* autooxidation; this process can mimic proton re-equilibration in systems that have pumped protons from inside to the bulk phase. 3. Proton re-equilibration following a pulse of cytochrome *c* or oxygen is multiphasic. The slowest phases are attributed to vesicle heterogeneity, some internal alkali being retained within vesicles of low intrinsic proton permeability. This can be overcome by the addition of either very low levels of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazine or high levels of valinomycin.

The overall oxidation of ferrocytochrome *c* by molecular oxygen (eqn. 1) involves an alkalization of the medium, when carried out at neutral pH and in the absence of ions capable of binding to the protein:

This is not true for all cytochromes. Thus the oxidation of cytochrome *aa*₃ (eukaryotic cytochrome oxidase) involves release of some bound protons (Wikström *et al.*, 1981) and the stoichiometry of eqn. (1) is not strictly observed. Nor is it true for cytochrome *c* at more alkaline pH values or in media where ion binding occurs. At alkaline pH, ferric cytochrome *c* undergoes a conformational

change involving proton release, probably from lysine-79 (Brandt *et al.*, 1966; Lambeth *et al.*, 1973). Oxidation would thus involve the process of eqn. (2):

Under conditions where ion binding can occur, ferrocytochrome *c* preferentially binds cations whereas ferricytochrome *c* preferentially binds anions (Margoliash *et al.*, 1970). If, therefore, the salt present is of a weak acid (X^+Y^-), the oxidation (in part) will follow eqn. (3):

Such anions, in addition to small species, may include protein and phospholipid surface groups.

When the reaction of eqn. (1) is catalysed by cytochrome oxidase, either in proteoliposomes or in intact mitochondria, the protons required for oxygen reduction are taken up internally (Wikström *et al.*, 1981; Mitchell, 1966, 1980). The appearance of external alkalinity is therefore slow unless a protonophore (uncoupler) is added to the

Abbreviations used: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazine; TMPD, *NNN'*-tetramethyl-*p*-phenylenediamine.

* To whom correspondence and reprint requests should be addressed.

† Present address: Department of Biochemistry, University of Western Ontario, London, Ontario N6A 4G5, Canada.

system. In the absence of protonophores but in the presence of an electrophoretic ionophore system such as valinomycin plus potassium, an initial acidification is seen in such systems (Krab & Wikström, 1978), of variable magnitude (Casey *et al.*, 1979; Proteau *et al.*, 1983). This acidification has been interpreted either as the result of a proton translocation from the mitochondrial or proteoliposomal interior to the outside (Wikström & Krab, 1979), or as the result of a process such as that in eqn. (3) (Mitchell & Moyle, 1983; Papa *et al.*, 1983a,b). These alternatives can be distinguished in proteoliposomes by: (i) demonstrating the disappearance of an equivalent amount of internal acid; (ii) quantifying the electrogenicity of the overall process (usually the countermovement of K^+ ions in the presence of valinomycin); or (iii) showing that the acidification correlates with ligand binding conditions and can occur when the vesicle membrane is no longer intact.

Hinkle (1973), Thelen *et al.* (1984) and Wrigglesworth & Nicholls (1979) have used trapped indicators to estimate internal alkalization. Such alkalization, however, includes the net effect of eqn. (3); demonstration of pumped protons therefore requires an accurate determination of trap volume or internal buffering power, which is not easy. Sigel & Carafoli (1980) have shown extra potassium uptake presumably compensating for 'pumped' protons. Again, such a demonstration demands accurate K^+ electrode calibration, and the low K^+ concentrations, favouring K^+ measurement, are unfavourable for effective charge compensation. Under such conditions stoichiometries are bound to be lower (Proteau *et al.*, 1983).

Papa *et al.* (1983a,b) have, in contrast, shown that extra H^+ release occurs under conditions favouring cytochrome *c* binding to the membrane, and also that this release is pH-dependent. They propose that H^+ release is consequent upon the rupture of protein salt bridges, as in eqn. (4):

In the present paper, we compare the proton release behaviour of proteoliposomes and of detergent-induced micelles of equivalent cytochrome *c* binding capability and with equivalent levels of oxidase activity. We find that, while the catalytic parameters and cytochrome *c* binding patterns are unaffected by lauryl maltoside addition to the proteoliposomes, both proton ejection and respiratory control are abolished over the same range of detergent concentrations. We also show that the scalar proton release accompanying cytochrome *c* binding at lower ionic strengths is uncoupler-insensitive and is not responsible for the anomalies seen at higher ionic strengths; we

attribute the latter to a slow collapse of respiration-induced internal alkalinity associated with a population of relatively proton-impermeable vesicles. Another paper (S. Shaughnessy, A. P. Singh, A. Chanady & P. Nicholls, unpublished work) will be devoted to a separate study of the rates and magnitudes of internal alkalization in such vesicles.

Materials and methods

Ox heart cytochrome oxidase was prepared from local slaughterhouse material as previously described (Brooks & Nicholls, 1982) and stock solutions containing about 0.25 mM-cytochrome *aa*₃ were stored at -70°C in 100 mM-sodium phosphate buffer, pH 7.4, containing 0.25% Tween 80. Asolectin was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.) in the form of 'L- α -phosphatidylcholine', 40%, type IV-S. In our hands this material is superior to acetone-washed asolectin from Associated Concentrates (Long Island, NY, U.S.A.) and is not improved by acetone washing. Cytochrome *c* (type VI) was also obtained from Sigma, as were sodium ascorbate and valinomycin. TMPD (as the chloride salt) was obtained from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.), and FCCP was a gift from Dr. P. G. Heytler (duPont Chemical Co., Wilmington, DE, U.S.A.). Lauryl β -D-maltoside was the product of Calbiochem-Behring (San Diego, CA, U.S.A.). Sodium dithionite was from BDH Chemicals (Poole, Dorset, U.K.). Sephadex G-25 and G-100 were either obtained via Sigma Chemical Co. or directly from Pharmacia Fine Chemicals AB (Uppsala, Sweden). All other chemicals were of the usual analytical grades.

Cytochrome *c* oxidase-containing proteoliposomes were prepared essentially according to Wrigglesworth & Nicholls (1979) and Proteau *et al.* (1983). Asolectin (0.5 g) was dispersed in 10 ml of 50 mM-potassium phosphate buffer, pH 7.4, with mechanical mixing. Stock cytochrome oxidase (0.1 ml, approx. 25 nmol) was then added, giving a phospholipid:protein ratio close to 80:1 (w/w). The mixture was further dispersed by hand, and then sonicated to clarity at 4°C (10–15 min) by using a Heat Systems Ultrasonics Sonicator model W375 with 0.5 cm probe, with a 30% pulsed mode duty cycle. The resulting solution was centrifuged for 10 min at 15000 rev./min in the A269 head of an IEC B-20A centrifuge to remove pelleted titanium and any undispersed lipid.

Oxidase activity was followed with an oxygen electrode (YSI Company, Yellow Springs, OH, U.S.A.) inserted in an appropriate reaction vessel (volume 4.1 ml) and connected to the necessary polarizing box, converter and recorder. Proton pulses were measured in a glass chamber (volume

8ml) with magnetic stirrer, into which a glass electrode (type G2222B or C; Radiometer Co., Copenhagen, Denmark) and calomel reference electrode (Radiometer type K701) were inserted. The latter was a double bridge type, in which the primary salt bridge contained saturated KCl, while the secondary salt bridge contained saturated NaCl (high ionic strength experiments) or half-saturated NaCl (low ionic strength experiments). The glass and reference electrodes were connected to a digital pH meter (Radiometer PHM 64) and recorder (Radiometer REA 100). Air-saturated media were prepared by bubbling filtered air through the appropriate solution at the reaction temperature for 2h. Ferrocyanochrome *c* was prepared by reduction with sodium ascorbate followed by removal of excess ascorbate on a Sephadex G-25 column.

In cytochrome *c* binding experiments (Table 1 below) two Sephadex G-100 columns (Brooks & Nicholls, 1982) were used. Equilibration and elution were carried out with 5mM-potassium phosphate buffer, pH 7.4, with or without 0.03% lauryl maltoside (see Table 1). Vesicles (1.5ml; approx. 3 μ M in cytochrome *aa*₃ and 1mg/ml in cytochrome *c*) were added and the fractions obtained were assayed spectrophotometrically with a DU-7HS UV-Vis spectrophotometer (Beckman Instruments, Irvine, CA, U.S.A.).

Protein contents were measured with the biuret method. Cytochrome oxidase concentration in solution and in vesicles was obtained by the method of Wrigglesworth & Nicholls (1979) using a $\Delta\Delta E$ value of 27mM⁻¹, 605 minus 630nm, reduced minus oxidized enzyme (Proteau *et al.*, 1983). Proton pulses were analysed according to Proteau *et al.* (1983).

Results

Effect of detergents

Fig. 1 compares the effects of lauryl maltoside and Triton X-100 on cytochrome *c* oxidase turnover in proteoliposomes. Triton X-100 is commonly used to solubilize membrane-bound systems. Fig. 1(a) shows the stimulation of turnover achieved by Triton addition to a vesicular enzyme system ($\blacktriangle$). At the same time, however, or at slightly lower Triton levels, the rate of turnover in presence of uncoupler diminishes ($\bullet$). In 0.1% Triton the maximum turnover is much less than that in intact vesicles. The inhibition of the uncontrolled activity occurs over the same concentration range as the release of controlled activity. The final activity level (80–100s⁻¹) is only about 30% of that obtainable under optimal conditions.

In contrast, Fig. 1(b) shows the stimulation of turnover achieved by lauryl maltoside, the deter-

Fig. 1. Effects of two detergents on cytochrome *c* oxidase activity in reconstituted vesicles

In each plot the ordinate shows oxidase turnover in μ mol of e⁻/s and the abscissa shows detergent concentration. The reaction vessel contained 13.2nM vesicular cytochrome *aa*₃, 4.9mM-ascorbate, 0.18mM-TMPD, 12.2 μ M-cytochrome *c* and 1.22 μ g of valinomycin/ml, and/or 1.22 μ M-FCCP where indicated. Total volume was 4.1 ml, and the medium was 50mM-potassium phosphate buffer, pH 7.4, at 30°C. (a) Triton X-100, (b) lauryl β -D-maltoside. $\blacktriangle$, control (detergent alone); $\blacksquare$, + valinomycin; $\bullet$, + valinomycin and FCCP.

gent introduced by Rosevear *et al.* (1980). Over a relatively narrow range of detergent concentrations (0.01–0.02% maltoside) both the controlled and uncontrolled systems are activated. Presumably micelle formation abolishes ionophore requirements in the controlled state; at the same time reduced cytochrome *c* is made available to the inward-facing oxidase molecules, approximately 45% of the total (Nicholls *et al.*, 1980). Fig. 1(b) thus shows that oxidase activity in presence of lauryl maltoside is almost identical with that of vesicular oxidase, and that the abolition of the vesicular topochemistry occurs synchronously with the eliciting of reactivity from cryptic (inverted orientation) enzyme. Other detergents, such as deoxycholate (results not shown), give results intermediate in character between those shown in Figs. 1(a) and 1(b). Tween-80, which is effective in maintaining an active state in isolated oxidase, is rather ineffective in releasing respiratory control.

Fig. 2 compares the actions of the two detergents

Fig. 2. Double reciprocal plots of cytochrome *c* oxidase activity as a function of cytochrome *c* concentration in the presence of two detergents

In each plot the ordinate shows $1/v$ (where v is oxidase turnover in $\mu\text{mol of e}^-/\text{s}$) and the abscissa shows $1/[S]$ (where $[S]$ =cytochrome *c* concentration). The reaction vessel contained 13.2 nM of vesicular cytochrome *aa*₃, 4.9 mM-ascorbate, 0.18 mM-TMPD, 1.22 μg of valinomycin/ml, and 1.22 μM -FCCP, in a total volume of 4.1 ml; the medium was 50 mM-potassium phosphate, pH 7.4, at 30°C. (a) Triton X-100, (b) lauryl β -D-maltoside. ●, control (no detergent); ■, 0.024% detergent; ▲, 0.122% detergent.

on apparent affinities for cytochrome *c* and V_{max} values. Inhibition of the uncontrolled activity by increasing Triton X-100 levels (Fig. 2a) is accompanied by a decrease in the apparent K_m value for cytochrome *c*. That is, Triton X-100 behaves as an uncompetitive inhibitor of the system, distinguishing in some way between enzyme and enzyme-cytochrome *c* complex. Activation of the uncontrolled activity by increasing lauryl maltoside concentrations, on the other hand (Fig. 2b), is accompanied by little or no change in apparent K_m value. Activation is expressed almost entirely as an

Table 1. Binding of cytochrome *c* to asolectin proteoliposomes in the presence and absence of lauryl maltoside: column chromatography

Proteoliposomes (1.5 ml, 2.95 μM in cytochrome *aa*₃) plus 0.1 ml. of approx. 1 mM-cytochrome *c* were placed on a G-100 column equilibrated at 25°C with 5 mM-potassium phosphate buffer pH 7.4, in the presence (b) or absence (a) of 0.03% lauryl maltoside. Fractions (1.1 ml) were examined after elution with phosphate buffer in the absence (a) or presence (b) of lauryl maltoside.

Expt.	Conditions	Fractions analysed*	Average ratio [cytochrome <i>c</i>]: [cytochrome <i>aa</i> ₃]
(a)	Control (intact proteoliposomes)	18–25	6.9
(b)	+ 0.03% lauryl maltoside (micelles)†	18–32	10.5

* The unbound cytochrome *c* was not eluted until fraction 40 was reached.

† Plus 30 mg of lauryl maltoside added with the proteoliposomes and cytochrome *c*.

effect on V_{max} , presumably reflecting only the appearance of binding sites associated with inverted enzyme molecules.

Sephadex G-100 chromatography of mixtures of vesicles and cytochrome *c*, in the presence and absence of lauryl maltoside, supports the idea that vesicle lysis is not accompanied by dramatic alterations in cytochrome *c* binding patterns. The results are summarized in Table 1.

As with catalytic activity, an increase in cytochrome *c* binding occurs as internal sites are exposed. Isolated cytochrome *aa*₃ at low ionic strength binds a maximum of 2 equivalents of cytochrome *c* under these conditions (Brooks & Nicholls, 1982). Asolectin liposomes themselves bind cytochrome *c* (Nicholls & Malviya, 1973) and the excess of 8 mol of cytochrome *c*/mol of cytochrome *aa*₃ in Table 1 presumably reflects such phospholipid-bound cytochrome.

The effect of lauryl maltoside upon oxygen-induced proton ejection by anaerobic proteoliposome suspensions was therefore examined. Fig. 3 illustrates some typical results obtained by using the apparatus described in the Materials and methods section. In the absence of lauryl maltoside or FCCP, a typical proton release pattern is seen (Fig. 3, trace a), followed by the expected net alkalinization predicted by eqn. (1). Addition of a small amount of lauryl maltoside diminishes the magnitude of the proton release as well as the net alkalinization (trace b), without affecting the rate of the latter (dependent on proton permeability) as measured by the half time for alkalinization. A

Fig. 3. Effect of lauryl maltoside on proton translocation by cytochrome *c* oxidase

The mixture used contained 3 ml of proteoliposomes ($0.35 \mu\text{M}$ -cytochrome aa_3 final concentration) plus 4 ml of medium ($45 \text{ mM-K}_2\text{SO}_4$ pH 7.4), 0.125 mM reduced cytochrome *c*, and $1.25 \mu\text{g}$ of valinomycin/ml. Trace *a* shows a control pulse; trace *b* shows a pulse in the presence of 0.125% maltoside; trace *c* shows a pulse in the presence of 0.125% maltoside and $1.25 \mu\text{M}$ -FCCP; trace *d* shows a pulse in the presence of $1.25 \mu\text{M}$ -FCCP; and trace *e* shows a pulse in the presence of $1.25 \mu\text{M}$ -FCCP (identical pulses were obtained at high levels of lauryl maltoside alone).

small amount of FCCP, sufficient to decrease the proton release by an equivalent amount (trace *d*), also increases the rate of realkalinization. At high levels of lauryl maltoside, or at high levels of FCCP in the presence or absence of lauryl maltoside (traces *c* and *e*), only alkalinization is seen. Low levels of lauryl maltoside, unlike FCCP, appear to create two families of cytochrome oxidase molecules, one still in intact vesicles, the other micellar in nature; FCCP makes all the vesicles present more proton-permeable without affecting accessibility or enzyme behaviour.

Some decline in net alkalinization appears to occur with lauryl maltoside micelles at low lauryl maltoside levels (Fig. 3, trace *b*). This resembles the effects seen by Papa *et al.* (1983*a*) at low ionic strength. We therefore carried out analogous experiments under such conditions which maximize cytochrome *c* binding (as used for the chromatographic experiments in Table 1).

Effect of low ionic strength

Fig. 4 illustrates the results of experiments carried out under conditions similar to those in Fig. 3, but with variations in the ambient ionic strength. Fig. 4(*a*) shows the results obtained in aerobic systems with pulses of reduced cytochrome *c*. Trace *a* shows the rather small proton pulse

obtained with a ferrocyanochrome *c* pulse at the usual ionic strength of $50 \text{ mM-K}_2\text{SO}_4$, abolished in the presence of FCCP (trace *b*). At low ionic strength ($5 \text{ mM-K}_2\text{SO}_4$), a much higher acidification pulse is seen (trace *c*), but this is no longer changed into alkalinization by FCCP (trace *d*). It is also insensitive to the oxidase inhibitors azide and cyanide (results not shown). A net acidification remains in all cases, as would be predicted if cytochrome *c* oxidation involved processes such as those in eqns. (3) and (4). Remarkably enough, similar results are obtained with liposomes prepared in the absence of cytochrome aa_3 (traces *e* and *f*). Cytochrome *c* binding, as demonstrated under analogous low ionic strength conditions (Table 1), can produce this result in the absence of enzyme or of cytochrome oxidation; it should be noted that the added ferrocyanochrome *c* (traces *a*–*d*) is oxidized within the mixing time ($< 2 \text{ s}$) as observed by eye or using an optical probe colorimeter (see the Materials and methods section).

However, as demonstrated by Wrigglesworth (1978) and others, cytochrome *c* can be oxidized slowly by an aerobic suspension of liposomes alone. Conversely, cytochrome *c* catalyses the aerobic peroxidation of unsaturated phospholipids by molecular O_2 (Singh *et al.*, 1985). This explains

Fig. 4. Effects of high and low ionic strength on ferrocytochrome *c*- and oxygen-induced pulses

(a) Ferrocytochrome *c* pulses into aerobic media. Each trace shows the effect of a 46 nmol pulse of cytochrome *c* (c^{2+}) into 8 ml of vesicle suspension at 30°C and pH approx. 7.4, in the presence of valinomycin (1.25 μ g/ml; all traces) and 1.25 μ M-FCCP (traces b, d and f). Traces a and b, 50 mM- K_2SO_4 (high ionic strength) with a final vesicular cytochrome *aa*₃ concentration of 0.64 μ M; traces c and d, 5 mM- K_2SO_4 (low ionic strength) with 100 mM-mannitol, 35 mM-sucrose and a final vesicular cytochrome *aa*₃ concentration of 1.42 μ M; traces e and f, medium as in traces c and d (low ionic strength) but the liposomes used contained no cytochrome *aa*₃ (approx 20 mg of lipid/ml). Upper traces (a, c, e) show results plus valinomycin; lower traces (b, d, f) show results plus valinomycin and FCCP. (b) O_2 -induced pulses into anaerobic media. Each trace shows the effect of a 0.1 ml (23.5 nmol of O_2) pulse of medium (O_2) into 8 ml of vesicle suspension at 30°C and pH 7.4, in the presence of valinomycin (1.25 μ g/ml, all traces) and 1.25 μ M-FCCP (traces b, d and f). Traces a and b, 50 mM- K_2SO_4 (high ionic strength) with proteoliposomal cytochrome *aa*₃ concentration of 0.6 μ M, 0.11 mM-cytochrome *c* and 0.125 mM-ascorbate. Traces c and d, 5 mM- K_2SO_4 (low ionic strength) with a proteoliposomal cytochrome *aa*₃ concentration of 0.53 μ M, 100 mM-mannitol, 35 mM-sucrose, 0.111 mM-cytochrome *c*, and 0.125 mM-ascorbate. Traces e and f, as in traces c and d, at low ionic strength, but with 1.0 μ M-cytochrome *c*, 5.0 mM-ascorbate and 94 μ M-TMPD present. Upper traces (a, c and e) show results plus valinomycin; lower traces (b, d and f) show results plus valinomycin and FCCP.

the slow collapse of the initial acidification in traces *e* and *f*, which occurs both in absence and presence of FCCP; it is due to the slow liposome-catalysed autoxidation of bound cytochrome *c*, which fortuitously under these conditions occurs at a rate similar to the rate of proton re-entry into the proteoliposomes (seen in traces *a* and *c*).

Fig. 4(b) shows that the differences between high and low ionic strength pulses are no longer seen when anaerobic oxygen pulses are employed. The oxygen-induced acidification seen at high ionic strength (trace *a*), abolished by FCCP (trace *b*) is also seen at low ionic strength (traces *c* and *d*). Moreover, the pulse at low ionic strength is also seen when cytochrome *c* is present at a much lower total concentration (traces *e* and *f*). Here, where ascorbate plus TMPD provides the source of net reductant, the initial proton pulse is the sum of the two processes of eqns. (5a) and (5b):

The final alkalinization (eqn. 6) is then only half that seen with ferrocyanide *c* alone (eqn. 1). The collapse of acidification proceeds more rapidly at low ionic strength (cf. traces *c* and *e* with trace *a*); this may reflect an increase in proton permeability or a decrease in effective buffering.

Fig. 5. Effect of oxygen on the realkalization following a ferrocyanide *c* pulse at low ionic strength

The initial acidification (apparent *n* value) is plotted against pulse number for experiments carried out as in Fig. 4(a) (▲). The percentage realkalization (as a return to the initial baseline) is also plotted against pulse number (●). Liposomes as in Fig. 4(a), traces *e* and *f* (protein-free) were incubated aerobically at pH 7.4, 30°C in 5mM-K₂SO₄/100mM-mannitol/35mM-sucrose, in the presence of valinomycin (1.25 μg/ml) and 0.435 mg of catalase (34000 units/mg). H₂O₂ (3.5 μmol) was added at the point indicated to replenish the oxygen in solution.

That the 'extra' acidification seen at low ionic strength reflects the initial binding of ferrocyanide *c*, and does not require its oxidation, is demonstrated by experiments in which a series of ferrocyanide *c* pulses are made to an initially aerobic liposome suspension (Fig. 5). At first the immediate acidification and final oxidation-dependent alkalinization are of similar magnitude. After seven pulses of about 6 μM-cytochrome *c*²⁺ each time, the oxygen-dependent slow alkalinization declines, while the initial acidification remains constant, up to at least twelve ferrocyanide *c* pulses. Peroxide addition in the presence of catalase (forming O₂ *in situ*) restores the oxidant in the system (lipid peroxide ?) whose formation is catalysed by bound cytochrome *c* and which acts to co-oxidize the bound *c*.

Parallel experiments with proteoliposomes, in which potassium ferricyanide, instead of O₂, was used to oxidize cytochrome *c*, gave no proton pulses (results not shown), under conditions where

Fig. 6. Effect of varying valinomycin concentration on the magnitude of oxygen-induced proton pulses (*n* values, *a*) and of the subsequent realkalization (*s* values, *b*)

Proteoliposomes (3.5 ml, final cytochrome *aa*₃ concentration 0.41 μM), in 50mM-K₂SO₄, 0.11mM-cytochrome *c*, 0.25mM-ascorbate plus valinomycin levels as indicated were pulsed with oxygen as in Fig. 4(b) at a pH value between 7.3 and 7.4, and a temperature of 28°C in a final volume of 8 ml. (*a*) *n* values (initial proton pulses); (*b*) *s* values (subsequent realkalization) (cf. Table 2). Addition of 1.25 μM-FCCP gave an *s* value of approx. 1.0 in all cases.

oxygen pulses clearly gave rise to an immediate acidification.

As reported by Casey *et al.* (1979) and discussed by Mitchell & Moyle (1983), the final realkalinization, presumably due to proton re-entry into the liposomes, shows complex kinetics and variable endpoints (cf. Fig. 3, trace *b*). Casey *et al.* (1984) have recently shown that the collapse of cytochrome *c*-induced H^+ pulses may be slower than that of HCl-induced gradients at low valinomycin concentrations. In our hands, the collapse of the pulse is commonly biphasic. The rate of the initial phase is usually identical to that of a control HCl pulse; the slow phase is much slower. Increasing valinomycin levels increase the proportion of the collapse occurring in the first, 'well-behaved', phase (Fig. 6). Fig. 6(*a*) shows that the elicited proton pulses are maximal at or below $0.5 \mu g$ of valinomycin/ml. Fig. 6(*b*) shows that the rapid realkalinization approaches the theoretical value of 1.0 (eqn. 1) only above $2.0 \mu g$ of valinomycin/ml. This requirement for a high level of valinomycin to secure return to the 'baseline' can be abolished by low levels of FCCP that are insufficient to eliminate the initial acid pulse (cf. traces *b*, *c*, and *d*, Fig. 3, and Casey *et al.*, 1979). Possible alternative interpretations of this finding are discussed below. Similar results were obtained with nonactin (results not shown).

Discussion

Table 2 summarizes the results obtained with both cytochrome *c* and oxygen pulses under the various conditions employed.

The following points may be noted: (*a*) net acidifications, which are FCCP-insensitive, occur only with cytochrome *c* pulses at low ionic strengths; (*b*) FCCP-sensitive O_2 -pulse-induced

acidifications are insensitive to ionic strength; (*c*) 's' values (net alkalizations) in the absence of FCCP may be less than 1, even when net FCCP-insensitive acidifications are not seen (including both O_2 and ferrocyanochrome *c* pulses at high ionic strengths); (*d*) FCCP addition, even at levels insufficient to abolish the initial acid pulse, secures $s=1$ values in all cases (cf. Casey *et al.*, 1979).

The 'shortfall' in alkalization discussed by Mitchell & Moyle (1983) thus has a double origin, in FCCP-insensitive net acidifications (seen only with cytochrome *c* pulses at low ionic strengths) and FCCP-sensitive biphasicity of proton backflow. As shown by Thelen *et al.* (1984) and confirmed by the data in Fig. 6, the biphasicity can also be abolished by increasing valinomycin levels. What is the reason for this phenomenon? Thelen *et al.* (1984) suggest that residual membrane potentials occur after the pulse, the effect of which is to oppose the return of protons to the liposomal interior. They do not, however, explain why such residual potentials are harder for valinomycin to dissipate than those created in the initial pulse which can only be seen in the presence of much lower levels of this ionophore. In any case, such residual potentials should be small and shortlived (Heinz *et al.*, 1981).

We wish to point out that following a pulse of either oxygen or cytochrome *c*, at least four aqueous compartments are present, each at a different pH value: (i) the bulk phase, acidified by the proton ejection; (ii) the internal phase of proton-pumping liposomes, made alkaline by pumping and by electron transfer; (iii) the internal phase of non-pumping enzymically active liposomes, made alkaline by electron transfer only; and (iv) the internal phase of liposomes devoid of active enzyme, at a neutral (commencing) pH.

The collapse of the acidity in (i) will depend on

Table 2. Summary of acidification and realkalinization produced by oxygen (anaerobic) and ferrocyanochrome *c* (aerobic) pulses at high and low ionic strength

Systems contained proteoliposomes ($0.3\text{--}0.6 \mu M$ -cytochrome a_{a3}), valinomycin ($1.25 \mu g/ml$), $1.25 \mu M$ -FCCP (for s' determination); $0.12 mM$ -cytochrome c^{2+} , and a trace of ascorbate ($\leq 0.12 mM$) in (*b*). Pulses of $5\text{--}6 \mu M$ -cytochrome c^{2+} in (*a*) and of $100 \mu l$ of medium into $8 ml$ total volume (i.e. $3 \mu M$ - O_2) in (*b*) were introduced. The pH maintained between 7.0 and 7.4 at $30^\circ C$. High ionic strength is $50 mM$ - K_2SO_4 ; low ionic strength is $5 mM$ - $K_2SO_4/100 mM$ -mannitol/ $35 mM$ -sucrose. n is the magnitude of proton pulse (H^+/e^-), s is the magnitude of final alkalization (OH^-/e^-), and s' is the magnitude of final alkalization in the presence of FCCP.

System	Pulse	Ionic strength	Parameter measured		
			+ Valinomycin		+ FCCP
			n	s	s'
(a) Aerobic	Cytochrome c^{2+}	High	0.18 ± 0.06	0.77 ± 0.44	0.87
		Low	0.72 ± 0.11	0.44 ± 0.06	—
(b) Anaerobic	O_2	High	0.39 ± 0.03	0.76 ± 0.11	1.02 ± 0.01
		Low	0.45 ± 0.02	0.87 ± 0.51	0.90 ± 0.02

the transmembrane gradients and permeabilities of (ii), (iii) and (iv). The collapses of the alkalinities in (ii) and (iii) will depend only upon their own permeabilities and gradients. Multiphasic behaviour is therefore to be expected. If some alkalized compartments are much tighter to protons than others, their decays will be much slower. Valinomycin at high concentrations (as in Fig. 6b) will act as a H^+ carrier rather than as a K^+ carrier. This is probably the explanation of the increased apparent permeability to H^+ ions seen at high valinomycin concentrations in the experiments of Biegel & Gould (1981). Valinomycin is capable of carrying protons, either as such or in the form of NH_4^+ ions [often present in protein-containing mixtures as a consequence of residual preparative $(NH_4)_2SO_4$ or deamidation processes].

We thus anticipate at least a triphasic decay process, involving first the disappearance of the externally 'pumped' protons into all the available buffering spaces, followed by two phases of equilibration of the internal alkalizations. If one of these latter is slow, the experimental consequences seen here and by Casey *et al.* (1979), Thelen *et al.* (1984) and discussed by Mitchell & Moyle (1983), will follow.

The results obtained using lauryl maltoside confirm the suggestion of Rosevear *et al.* (1980) that this is close to an 'ideal' detergent for cytochrome oxidase, that is one in which the enzyme behaves identically to its membrane-associated counterpart, except for transmembrane (topochemical) functions. The proportional increase in activity that results from the dispersal of the proteoliposomes by lauryl maltoside (Fig. 1b) is identical to the proportional increase in cytochrome *a* reduction obtained by addition of TMPD to an ascorbate-cytochrome *c* reduced system. This then resolves the apparent discrepancy between the latencies reported by Wrigglesworth & Nicholls (1979) and by Carroll & Racker (1977). Even the cytochrome *c*-binding properties change in the same way (Table 1) and the apparent K_m for cytochrome *c* oxidation is unaffected (Fig. 2b).

However, proton ejection is abolished under conditions where other parameters remain unchanged. This seems to us to be convincing evidence that such ejection is a topochemical event. Other experiments, in which internal vesicle pH is monitored (S. Shaughnessy, A. P. Singh, A. Chanady & P. Nicholls, unpublished work), are however needed to confirm this. Unlike low levels of FCCP, which partially abolish respiratory control in all vesicles in a given sample, low levels of lauryl maltoside completely abolish proton ejection in part of the sample while the remainder continues to show normal control characteristics (Fig. 3). At the same time, the anomalous pattern

of proton re-equilibration is retained or exaggerated. We conclude that lauryl maltoside has an 'all-or-none' effect on vesicles of the several possible types mentioned above. An individual enzyme molecule is either in a fully intact vesicle or in a micelle; families of vesicles showing different initial proton permeabilities may also show different lauryl maltoside sensitivities. Further experiments may be needed to quantify these effects and to study further the detailed behaviour of the oxidase and its cytochrome *c* binding sites in the presence of 'friendly' detergents such as lauryl maltoside.

The exact stoichiometry of proton translocation by the oxidase, its dependence on subunit III and its control, remain open questions. In most of our experiments, only about one proton is seen to be ejected per atom of oxygen taken up, compared with the two protons consistently shown by Wikström *et al.* (1981). Perhaps, as suggested above, some of our enzyme, as prepared, lacks a functional 'pump' (subunit III) yet is still capable of incorporation into vesicles demonstrating respiratory control (cf. the effects of heat in Sone & Nicholls, 1984). That the oxidase is capable of proton translocation across the liposomal membrane does, however, seem to be proven.

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