The proteoliposomal steady state

Effect of size, capacitance and membrane permeability on cytochrome-oxidase-induced ion gradients

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1. The flux pathways for H^+ and K^+ movements into and out of proteoliposomes incorporating cytochrome *c* oxidase have been investigated as a function of the electrical and geometrical properties of the vesicles. 2. The respiration-induced pH gradient (ΔpH) and membrane potential ($\Delta\psi$) are mutually dependent and individually sensitive to the permeability properties of the membrane. A lowering or abolition of $\Delta\psi$ by the addition of valinomycin increased the steady-state level of ΔpH . Conversely, removal of ΔpH by the addition of nigericin resulted in a higher steady-state $\Delta\psi$. 3. Vesicles prepared by sonication followed by centrifugation maintained similar pH gradients at steady state to those in vesicles prepared by dialysis, although the time taken to reach steady state was longer. Higher pH gradients can be induced in non-centrifuged sonicated preparations. 4. No significant differences were found in H^+ and K^+ permeability between proteoliposomes prepared by dialysis or by sonication. The permeability coefficient of the vesicle bilayers for H^+ was $6.1 \times 10^{-4} \text{ cm} \cdot \text{s}^{-1}$ and that for K^+ was $7.5 \times 10^{-10} \text{ cm} \cdot \text{s}^{-1}$. An initial fast change in internal pH was seen on the addition of external acid or alkali, followed by a slower, ionophore-sensitive, change. The initial fast phase can be increased by the lipid-soluble base dibucaine and the weak acid oleate. In the absence of ionophores, increasing concentrations of oleate increased the rate of H^+ translocation to a level similar to that seen in the presence of nigericin. Internal alkalization could also be induced by oleate upon the addition of potassium sulphate. 5. The initial, pre-steady-state and steady-state ΔpH and $\Delta\psi$ changes can be simulated using a model in which the enzyme responds to both ΔpH and $\Delta\psi$ components of the protonmotive force. At steady state, the electrogenic entry of K^+ is countered by electroneutral exit via a K^+/H^+ exchange. 6. The permeability coefficient, P_H , calculated from H^+ flux under steady-state turnover conditions, was approx. 100 times higher than the corresponding 'passive' measurements of P_H . Under conditions of oxidase turnover, the vesicles appear to be intrinsically more permeable to protons.

INTRODUCTION

Biochemical reactions in small compartments, whether micellar (Martinek *et al.* 1986) or membrane-bound (Casey, 1984), can be controlled and limited by the effects of compartment size and electric field (Wrigglesworth, 1985; Grover *et al.*, 1985; Walde *et al.*, 1988). Substrate availability will depend on the volume which is accessible to the enzyme as well as on the rates of substrate entry and product exit from the compartment (Luisi *et al.*, 1988). The translocation of charged species in and out of a membrane-bound compartment may be limited by the electrical capacitance and internal buffer concentrations (Wrigglesworth, 1985). Under steady-state conditions, constraints will be placed on the flux pathways for ionic species (Nicholls *et al.*, 1987). The large area/volume ratio inside the compartment may also result in adsorption/activity effects on entrapped molecules, including water (Walde *et al.*, 1988). All of these effects may have consequences on the functioning and control of enzymes *in vivo*. In addition, this is an area where uncertainties are limiting the use of reconstituted vesicular systems in many practical applications (Wrigglesworth, 1988).

Proteoliposomes incorporating a transmembrane enzyme, cytochrome oxidase, provide a convenient test bed for investigations into some of these problems. This enzyme is capable of generating an electrochemical gradient of protons across the vesicle membrane, which, in turn, can exert a control

effect on turnover (Hinkle *et al.*, 1972). Both the membrane potential ($\Delta\psi$) and the pH gradient (ΔpH), the two components of the electrochemical gradient, can be manipulated using specific ionophores, but it is not clear to what extent they are equivalent in their effects on the enzyme (Gregory & Ferguson-Miller, 1988). It is also not clear to what degree the size, geometry and capacitance of the vesicle can modify the flux properties of the reconstituted system. For example, respiratory control has been shown to vary inversely with vesicle size in some preparations (Wrigglesworth & Nicholls, 1979; Sarti *et al.*, 1989). Nevertheless, cytochrome-oxidase-containing vesicles prepared by sonication and by detergent dialysis both give respiratory control values of around 6, despite having different average diameters and an order of magnitude difference in electrical capacitance (Wrigglesworth, 1985). The flux pathways for protons and other cations during steady-state turnover are also not certain (Singh & Nicholls, 1984). Work with proteoliposomes has led to suggestions of 'intrinsic uncoupling' or 'slip' with regard to H^+ translocation within the enzyme, in contrast to 'extrinsic uncoupling' caused by proton leakage through the lipid bilayer (e.g. see Malmstrom, 1989).

The present experiments were aimed at quantifying the effects of vesicle size and capacitance on the pre-steady-state and steady-state values of ΔpH and $\Delta\psi$ following the initiation of turnover in cytochrome oxidase proteoliposomes. Measurements of proton permeability under passive and active (turnover) conditions

Abbreviations used: ΔpH , $\Delta\psi$, transmembrane pH gradient and potential respectively; DiSC₃-5, 3',3'-dipropylthiadicarbocyanine; TMPD, *NNN'*-tetramethyl-*p*-phenylenediamine; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazine.

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has allowed for a quantitative measurement of H^+ and K^+ flux pathways during the steady state. The results provide evidence for weak acid/base components in the lipid bilayer which are able to catalyse an electroneutral H^+/K^+ exchange, and also suggest the existence of an intrinsic H^+ leak pathway through the oxidase under turnover conditions.

MATERIALS AND METHODS

Cytochrome *c* oxidase was prepared from ox heart essentially according to Kuboyama *et al.* (1972), with Tween-80 substituting for Emasol. The final preparations (approx. 300 μM with a total haem *a*/protein ratio between 9 and 12 nmol/mg) were stored at $-75^\circ C$ in 100 mM-potassium phosphate buffer, pH 7.4, containing 0.1% Tween-80 until required.

Reconstituted proteoliposomes were prepared by detergent dialysis and by sonication. For the detergent dialysis method, 0.25 g of dry asolectin (L- α -phosphatidylcholine, Sigma type IV-S) was dispersed by mixing in 5 ml of 100 mM-Hepes buffer, pH 7.2; 0.1 g of sodium cholate was then added and the sample was sonicated for 2 min. Cytochrome oxidase was then added to a final concentration of approx. 4 μM and, after mixing, the sample was dialysed against 100 vol. of 100 mM-Hepes buffer, pH 7.2, with three changes of medium over a period of 2 days. For vesicles incorporating pyranine (8-hydroxy-1,3,6-pyrene trisulphonate, trisodium salt; Molecular Probes Inc., Eugene, OR, U.S.A.) as a monitor of internal pH, the dye was added to the detergent/phospholipid/protein mixture before dialysis to a final concentration of 5 mM. The dialysing medium also contained dye to the same concentration. After dialysis, excess dye was removed from the samples by gel filtration on a column of Sephadex G-75 equilibrated with 100 mM-Hepes buffer, pH 7.2. Vesicles were prepared by sonication according to Proteau *et al.* (1983). Prolonged sonication (more than 15 min in a pulse sonicator) has to be avoided, as this results in oxidase inactivation. Centrifugation (20000 *g*, 15 min) was routinely done for all sonicated preparations to remove any tungsten particles arising from the probe and also to remove the larger vesicles and any non-dispersed lipid aggregates. Excess dye was removed by gel filtration as described above. DiSC₃-5 (3',3'-dipropylthiadicarbocyanine, a gift from Dr. Alan Waggoner, Carnegie-Mellon University, Pittsburgh, PA, U.S.A.) was used to monitor $\Delta\psi$ and was added directly to the vesicle suspension in the fluorescence cuvette to a final concentration between 1 and 5 μM .

Fluorescence changes were monitored using a multipurpose cuvette system as described previously (Singh *et al.*, 1985). Pyranine excitation was at 460 nm with emission monitored at 90° to the excitation beam using an Oriel long-pass filter (50% cut-off at 480 nm). DiSC₃-5 excitation was at 622 nm with emission monitored using a 670 nm long-pass cut-off filter. Spectrophotometric measurements of changes in pyranine absorbance were monitored at 455 nm minus 516 nm using an Aminco DW-2 (dual-wavelength) instrument.

Cytochrome *c* (horse heart; Sigma type VI), sodium ascorbate, TMPD (*NNN'*-tetramethyl-*p*-phenylenediamine), Mops, oleic acid, dibucaine and valinomycin were obtained from Sigma Chemical Co. FCCP [carbonyl cyanide *p*-(trifluoromethoxy)-phenylhydrazone] was a gift from Dr. P. G. Heytler, du Pont Co., Wilmington, DE, U.S.A., and nigericin was obtained from Eli Lilly & Co., Indianapolis, IN, U.S.A.

Kinetic modelling of the vesicular steady state was carried out by numerical integration of the differential equations described in the Discussion section using the program STELLA 2.10 (High Performance systems Inc., Lyme, NH, U.S.A.) in the Runge-Kutta fourth-order mode on an Apple-Macintosh SE computer.

Fig. 1. Effect of low ionophore concentrations on the respiration-dependent changes in ΔpH and $\Delta\psi$ in cytochrome oxidase proteoliposomes

Changes in (a) ΔpH and (b) $\Delta\psi$ were monitored in proteoliposomes, prepared by dialysis, containing entrapped pyranine (5 mM) in 0.1 M- K^+ -Hepes, pH 7.2. For measurement of ΔpH , proteoliposomes (0.9 mg of lipid; 0.11 nmol of *aa₃* accessible to external cytochrome *c*) were suspended in 2.8 ml. A 5 times lower concentration of proteoliposomes was used for measurement of $\Delta\psi$, with a cytochrome *c* concentration of 0.65 μM . Turnover was initiated by the addition of ascorbate (1.7 mM) plus TMPD (49 μM). The indicated catalytic-centre activities were calculated from a simultaneous monitor of O_2 consumption using an oxygen electrode.

RESULTS

Initiation of turnover of externally facing (cytochrome-*c*-binding site facing out) cytochrome oxidase in sonicated proteoliposomes generates ΔpH (inside alkaline) and $\Delta\psi$ (inside negative) across the liposome bilayer (Fig. 1). For convenience, the traces can be divided into three main phases. First, an initial fast rise (< 5 s) in $\Delta\psi$ is accompanied by a small but fast rise in internal alkalization. In the second phase, ΔpH continues to rise, but at a much slower rate (minutes). This increase in internal alkalization is accompanied by a correspondingly slow fall in $\Delta\psi$. Finally, both ΔpH and $\Delta\psi$ level off at steady state. The magnitude of the ΔpH at steady state is around 0.3 pH units for these sonicated vesicles, and $\Delta\psi$ has been measured under comparable conditions by Singh & Nicholls (1985, 1986) as being between 80 and 150 mV (negative inside), the exact value depending on the method of measurement.

As shown previously, using Phenol-Red-loaded proteoliposomes (Hinkle, 1973; Wrigglesworth, 1978; Wrigglesworth & Nicholls, 1979), the internal alkalization is sensitive to ionophores and uncouplers. Corresponding changes in $\Delta\psi$ also occur (Singh *et al.*, 1985). Addition of the electroneutral K^+/H^+ exchanger nigericin abolishes ΔpH whilst increasing $\Delta\psi$. Increasing the electrogenic K^+ flux by valinomycin abolishes $\Delta\psi$

and increases ΔpH . A combination of both ionophores at high enough concentrations abolishes both membrane potential and internal alkalization. In the present case (Fig. 1), it is important to note that combinations of electrogenic and electroneutral ionophores at low concentrations can be used to manipulate the steady-state levels of ΔpH and $\Delta\psi$. Increasing the electroneutral K^+/H^+ exchange capability of the membrane by the addition of a small amount of nigericin lowers ΔpH almost to zero (Fig. 1a). Little change is seen in oxidase turnover. The new steady-state value of ΔpH is now very sensitive to the addition of valinomycin, which causes a relatively large rise in ΔpH . Conversely, the addition of a small amount of valinomycin lowers the steady-state level of $\Delta\psi$ with an inhibitory effect on oxidase turnover (Fig. 1b). The subsequent addition of nigericin increases both turnover and $\Delta\psi$. It is apparent that ΔpH and $\Delta\psi$ are mutually dependent and individually sensitive to the permeability properties of the membrane. These properties in turn are very sensitive to trace amounts of electrogenic and electroneutral ion carriers present in the system.

It was found that lower concentrations ($<0.5 \mu\text{g} \cdot \text{ml}^{-1}$) of valinomycin give a higher steady-state ΔpH than higher concentrations of the ionophore (Fig. 2). These lower valinomycin concentrations still decrease $\Delta\psi$ (Fig. 1b). The initial rate of internal alkalization following the initiation of turnover is increased by more than 10-fold at these concentrations (Fig. 2). However, somewhat surprisingly, the decay phase on cessation of turnover is not significantly affected. It is only at the higher concentrations of valinomycin that the decay phase begins to match the rise phase. We attribute these effects to the known affinity of the ionophore for H^+ as well as K^+ (Seigneuret & Rigaud, 1986; Hodarnau *et al.*, 1986). At low concentrations of valinomycin and high concentrations of potassium, the ionophore predominantly affects K^+ flux. At the higher concentrations, an increasing effect on H^+ translocation occurs. From the results shown in Fig. 2, we therefore conclude that K^+ flux is not rate-limiting to H^+ movement following anaerobiosis, since addition of valinomycin at low concentrations has little

Fig. 2. Effect of valinomycin concentration on respiration-dependent changes in internal pH

Vesicles containing entrapped pyranine in 0.1 M- K^+ -Hepes were suspended in 2.8 ml of K^+ -Mops (5 mM), pH 7.2, plus KCl (100 mM). Turnover was initiated by the addition of ascorbate (7 mM), TMPD (0.26 mM) and cytochrome *c* (3.5 μM). (a) Control; (b) plus valinomycin.

Fig. 3. Effect of valinomycin on passive H^+ efflux from liposomes in the presence and absence of FCCP

Liposomes containing entrapped pyranine but without incorporated oxidase were prepared as described in the Materials and methods section. A 0.2 ml portion of suspension (0.4 mg of lipid) was added to 2.8 ml of K^+ -Hepes (0.1 M), pH 7.0, and a pH gradient was imposed by the addition of NaOH as indicated.

effect on the decay rate. This conclusion was also reached by Nichols & Deamer (1980), using liposomes prepared by the ether evaporation technique. We have directly tested this result in the present type of liposome using an externally added pulse of alkali to impose a pH gradient (Fig. 3). Low concentrations of valinomycin can be seen to have little effect on the subsequent rate of internal alkalization. However, the same low level of valinomycin does affect the rate in the presence of FCCP. In this case, FCCP has increased proton flux to the extent that K^+ counter-ion movement has become rate-limiting, and thus the proton flux is now sensitive to valinomycin. The protonophoric properties of valinomycin, although known (Seigneuret & Rigaud, 1986; Hodarnau *et al.*, 1986), are not generally appreciated and can lead to error in experimental interpretation if the ionophore is used at high concentrations.

Effect of vesicle size on ΔpH and $\Delta\psi$

Both dialysed and sonicated preparations of proteoliposomes generate ΔpH and $\Delta\psi$, and both preparations exhibit respiratory control (an increase in turnover in the presence of valinomycin and FCCP) with a control ratio of around 6. However, the geometry and size of vesicles in the two preparations differs. Sonication produces a mixture of uni- and oligo-lamellar vesicles which are heterogeneous in size (diameter 30–200 nm), in contrast with the more uniform distribution of smaller-sized unilamellar vesicles (diameter 30–50 nm) found in dialysed preparations (Wrigglesworth, 1985; Cooper & Nicholls, 1990). The number of larger vesicles in the sonicated preparation can be decreased by the centrifugation and gel-filtration procedure used in the present work. Some differences between the two types of preparation can be seen in the rate of formation of steady-state ΔpH (Fig. 4). The non-centrifuged sonicated vesicles take longer to reach steady state than do the dialysed vesicles or the sonicated vesicles subjected to centrifugation. The final ΔpH level also seems to be higher if the sonicated sample is not centrifuged after preparation. Centrifugation usually increases respiratory control by a small extent (from 4–5 to 5–6), but does appear to lower the ability of the vesicles to maintain a higher steady-state ΔpH . One explanation may be that the larger vesicles, which are removed by

Fig. 4. Effect of vesicle size on respiration-dependent pH changes in cytochrome oxidase proteoliposomes

Proteoliposomes ($0.05\text{--}0.1\ \mu\text{M}$ aa_3 accessible to external cytochrome c) were suspended in $0.1\ \text{M}$ K^+ -Hepes buffer, pH 7.2, containing $2.7\ \mu\text{M}$ cytochrome c at 30°C . Turnover was initiated by the addition of ascorbate ($1.7\ \text{mM}$) plus TMPD ($49\ \mu\text{M}$). Vesicle preparations with different proteoliposome size distributions were prepared by sonication and dialysis as described in the text. (a) Sonicated preparation with no centrifugation (diameter range $30\text{--}200\ \text{nm}$); (b) centrifuged sonicated preparation (diameter range $30\text{--}80\ \text{nm}$); (c) dialysed preparation (diameter range $25\text{--}50\ \text{nm}$).

centrifugation, contain on average more oxidase molecules per vesicle than the smaller vesicles (Wrigglesworth *et al.*, 1987). The flux of proton removal per vesicle would be higher in these vesicles compared with vesicles with less oxidase incorporation. For the same turnover per oxidase molecule, the larger vesicles would then be capable of generating a higher ΔpH than the smaller vesicles. The fact that this preparation shows a slightly lower respiratory control than the centrifuged sample may be a result of uncoupled turnover by heterogeneous lipid/oxidase aggregates which can be removed by centrifugation.

Permeability properties of sonicated and dialysed vesicles

No significant differences were found in the membrane permeability to H^+ between proteoliposomes prepared by sonication and those prepared by dialysis. This was assessed by measuring the decay of a pH gradient imposed by the external addition of KOH to vesicles prepared by dialysis and sonication (Fig. 5). The rate of imposed ΔpH decay in the presence of FCCP, where proton permeability is no longer rate-limiting, has a value approximately the same as that found in the presence of a high concentration of valinomycin. As expected, the presence of nigericin resulted in an immediate pH equilibration across the membrane (results not shown).

An additional effect seen in Fig. 5 is that the vesicles show some initial rapid pH change (extrapolation of broken lines) after the addition of alkali. Clement & Gould (1981) have attributed the rapid pH change after the external addition of acid to phospholipid vesicles to an initial electrically uncompensated H^+ influx. The subsequent generation of a transmembrane electric potential (inside positive) then limits further H^+ movement to a slower proton-counter-ion exchange. In the present experiments using externally added alkali or acid, a proportion of the initial change in pyranine fluorescence is undoubtedly due to some of the probe being present outside the vesicles. This is especially pronounced in vesicles stored (days) after the initial gel-filtration stage. Passing the preparation through the filtration column a second time does lower the size of the initial change, although the effect cannot be eliminated by this procedure.

An initial rapid pH change can also be seen under 'active' conditions, where ΔpH is a result of oxidase turnover (e.g. see

Fig. 5. Passive H^+ permeability of proteoliposomes prepared by sonication and by dialysis

Proteoliposomes, prepared by sonication ($\bullet$, $\blacktriangle$) or by dialysis ($\circ$, $\triangle$, $\square$) and containing entrapped pyranine ($5\ \text{mM}$) were suspended in $0.1\ \text{M}$ K^+ -Hepes buffer, pH 7.3, at an aa_3 concentration of approx. $0.1\ \mu\text{M}$. A pH gradient of approx. 0.2 units was imposed by the addition of KOH, and changes in pyranine fluorescence were monitored as described in the text. Valinomycin ($\blacktriangle$, $\triangle$) and FCCP ($\square$) were used at $1.8\ \mu\text{g}\cdot\text{ml}^{-1}$ and $1.8\ \mu\text{M}$ respectively.

Fig. 6. Comparison of dibucaine and oleate as modifiers of fast and slow H^+ permeation through proteoliposomal membranes

Proteoliposomes prepared by sonication and containing entrapped pyranine ($5\ \text{mM}$ in $50\ \text{mM}$ potassium phosphate, pH 7.4) were suspended in $50\ \text{mM}$ potassium phosphate. Changes in pyranine absorbance were monitored following the rapid addition of KOH to the suspension. (a) Control; (b) plus dibucaine ($80\ \mu\text{M}$); (c) plus oleate ($80\ \mu\text{M}$); (d) plus FCCP; (e) dibucaine plus FCCP; (f) oleate plus FCCP.

Fig. 1). The rapid pH change occurs at the same time as the initial rise in $\Delta\psi$ and is generally larger in the smaller (dialysed) vesicles (Fig. 4).

We have investigated further some of the conditions that affect

Fig. 7. Effect of oleate on H^+ permeation through proteoliposomal membranes following the external addition of (a) NaOH and (b) K_2SO_4 .

Liposomes (approx. 4 mg of lipid) prepared by dialysis and containing entrapped pyranine were suspended to a final volume of 3 ml in K^+ -Hepes (100 mM), pH 7.0. NaOH (a) or K_2SO_4 (b) were added as indicated and the changes in pyranine absorbance (ΔpH) were monitored as in Fig. 6. Trace a, control; b, 1.67 mM-oleate; c, 2 mM-oleate; d, 3.33 mM-oleate. Oleate was added as an ethanolic solution at 3–5 min before the addition of NaOH or K_2SO_4 . Nigericin ($0.66 \mu g \cdot ml^{-1}$) when added in the absence of oleate gave the same response as shown in traces d.

H^+ flux under passive conditions. It has been found that the presence of lipid-soluble weak-base or weak-acid components can affect both the initial fast phase and the subsequent slower pH equilibration rate. Fig. 6 shows the effects of adding the weak base dibucaine and the weak acid oleate on H^+ exit following external alkali addition to the vesicles. These compounds increase the rate of H^+ equilibration, in both the absence and the presence of FCCP. Dibucaine can also be seen to affect the magnitude of the initial fast phase.

Fig. 7(a) shows that increasing concentrations of oleate lead to an increase in the rate of alkalinization to a level similar to that seen in the presence of nigericin. Increases in the fast phase also occur. For both oleate and dibucaine, the increase in the fast phase was found to be stoichiometric with added compound (results not shown). Internal alkalinization can also be observed by induction of a K^+ gradient. External addition of K^+ (as sulphate) in the presence of oleate induces a ΔpH (Fig. 7b). The H^+ efflux rates following a K^+ or OH^- pulse in the presence of high oleate concentrations approach those seen in the presence of

nigericin. The results suggest that oleate can catalyse K^+ as well as H^+ translocation. This would be feasible if both the free acid and the potassium soap of oleate were membrane-permeable, allowing electroneutral K^+/H^+ cycling across the membrane.

A simple detergent action of oleate on the vesicles is precluded by the fact that the vesicles are able to generate a pH gradient on K^+ addition. Membrane integrity would appear to be maintained. This was not found to be the case in the presence of 25 mM-Tween-80 (results not shown). The relationship between the amount of oleate added and the actual concentration in the membranes is unknown. For example, Simpson *et al.* (1988) found that preincubation of intestinal brush-border membrane vesicles for 30 min at 37 °C was necessary to incorporate 50 % of added oleate. Incorporation is likely to be much lower under the present conditions, where oleate was added 3–5 min before K^+ or OH^- addition.

DISCUSSION

In the presence of an impermeant substrate such as ascorbate or ferrocytochrome *c*, cytochrome oxidase incorporated into lipid vesicles will generate a membrane potential (internally negative) (Jasaitis *et al.*, 1972; Hinkle *et al.*, 1972; Drachev *et al.*, 1976; Casey *et al.*, 1984; Singh & Nicholls 1984, 1985, 1986) and an internal alkalinization (Hinkle, 1973; Wrigglesworth, 1978; Wrigglesworth & Nicholls, 1979). From the present results, it is clear that $\Delta\psi$ and ΔpH are interdependent in magnitude and rate of change. These quantities, in turn, depend on oxidase turnover and the geometrical, permeability and electrical characteristics of the vesicle. To separate effects of the oxidase from those due to the properties of the vesicle needs a quantitative analysis of the flux characteristics of the whole system.

Size, capacitance and membrane permeability

A quantitative analysis of the changes in ΔpH and $\Delta\psi$ requires some knowledge of the physical properties of the vesicular system. Table 1 presents some parameters for sonicated and dialysed proteoliposomes based on the size measurements made by Wrigglesworth (1985). The average size for the sonicated preparations used in the present work was decreased by centrifugation and gel filtration, but the two extremes shown give some estimate of the range of physical parameters that can be encountered. In dialysed preparations, an efflux of 50 H^+ equivalents would lead to a ΔpH of around 0.3. With an oxidase turnover around $30 s^{-1}$, this should take place in just less than 1 s, assuming that 2 H^+ equivalents are removed from the interior by the oxidase for every electron equivalent passed to oxygen. Even with the larger sonicated vesicles, a rise of 0.3 ΔpH should take place within 16 s. These time periods are seen in the presence of valinomycin when $\Delta\psi$ is zero (see for example the rate of alkalinization following the addition of valinomycin in Fig. 2). However, in the absence of valinomycin, longer periods occur. Under these conditions, $\Delta\psi$ will be present and have a strong effect on the electrogenic entry of H^+ , probably in a non-ohmic manner (Krishnamoorthy & Hinkle, 1984; O'Shea *et al.*, 1984). In the absence of the ionophore, a similar rate of removal of H^+ by the enzyme will be opposed by a much larger electrogenic entry of H^+ . The rates of internal alkali generation under these conditions are therefore slower, up to 10–15 times according to the present experiments.

The values of electrical capacitance calculated (see Table 1) allow for a quantitative estimate of ΔpH and $\Delta\psi$ in the initial electrogenic phase following initiation of oxidase turnover. The electrogenic separation of 56 charges by the oxidase (seven turnovers) would lead to the generation of a 100 mV membrane potential in the larger vesicles. A similar potential could be

Table 1. Some geometric properties of small vesicles prepared by sonication and by dialysis

The values for average diameter of the two preparations and the oligomeric structure of sonicated preparations are based on measurements reported by Wrigglesworth (1985).

Parameter	Sonicated		Dialysed
Average diameter	80 nm	($\approx 0.5 \mu\text{F}/\text{cm}^2$)	30 nm
Structure	Oligolamellar		Unilamellar
'Accessible' volume	1×10^{-19} litre (1 mM $\equiv$ 60 molecules)		5×10^{-21} litre (1 mM $\equiv$ 3 molecules)
Geometric capacitance ($C = \frac{2.5ab}{b-a} \text{ cm}$)	9×10^{-17} F/vesicle (100 mV $\equiv$ 56 ions)		1×10^{-17} F/vesicle (100 mV $\equiv$ 6.3 ions)
Internal pH changes (100 mM-Hepes, pH 7.5)	$0.5 \Delta\text{pH} \equiv 1500 \text{ H}^+$		$0.5 \Delta\text{pH} \equiv 75 \text{ H}^+$

generated in the smaller dialysed vesicles by just one oxidase turnover. The equivalent transmembrane movement of H^+ out of the vesicles would create initial electrogenic ΔpH values of approx. 0.02 and 0.04 for sonicated and dialysed vesicles respectively. Values of around 0.02–0.05 ΔpH for the initial fast phase under active turnover conditions can be seen in the present work.

Under passive conditions where external acid or alkali are added to artificially create a ΔpH of around 0.2, the induced $\Delta\psi$ will be much lower (around 12 mV), and yet small but significant changes in ΔpH can still be seen (Fig. 5) even after additional gel filtration to remove external dye. Similar initial fast changes in ΔpH on the addition of acid to liposomes were reported by Clement & Gould (1981). These changes were attributed to a rapid electrically uncompensated H^+ movement. From the present calculations, it would appear that the changes in ΔpH are too great for this explanation, and either the values of capacitance are in error by an order of magnitude, or some other, non-electrogenic, mechanism for fast H^+ movement is present in the vesicle membrane. The calculated value of capacitance/ cm^2 for the vesicles of around $0.5 \mu\text{F}$ is in general agreement with calculated and measured values of capacitance for phospholipid bilayers and biological membranes in general (Pauley *et al.*, 1960; Montal, 1976). If this value is accepted, then one possible

mechanism for the initial fast pH change on the addition of external acid or alkali could be based on a non-electrogenic H^+ movement, as suggested by the experiments of Hope & Cullis (1987) and Gutknecht (1987), and illustrated in Fig. 8. Some H^+ translocation is proposed to be catalysed by association with weak-acid (Fig. 8a) or weak-base (Fig. 8b) components in the membrane. Fast translocation occurs with the uncharged species, AH for associated acid and B for unassociated base. The corresponding charged species, A^- and BH^+ , have relatively low permeability rates through the membrane and depend on compensating electrogenic ion movements. The addition of external alkali will deprotonate both external acid and base. However, only the uncharged base will be able to cross the membrane and catalyse a fast stoichiometric uptake of H^+ from the interior. Conversely, on the addition of external acid, a fast stoichiometric uptake of H^+ will be catalysed by the membrane-associated weak-acid components. It has been shown that a transmembrane migration of stearylamine and stearic acid can be rapidly induced in response to transmembrane pH gradients (Hope & Cullis, 1987; Hope *et al.*, 1989). Gutknecht (1987) has also shown that weakly acidic contaminants in phospholipid preparations can act as proton carriers at neutral pH, and the apparent pK_a values of fatty acids and fatty amines in phosphatidylcholine bilayers have been measured as 7.2 and 9.5 respectively by Ptak *et al.* (1980).

Fig. 8. H^+ translocation across the proteoliposomal membrane catalysed by (a) weak-acid and (b) weak-base cycles

In the presence of a lipid-soluble weak acid, addition of H^+ to one side of the membrane results in the formation and fast translocation of the conjugated species (HA) to the other side. Dissociation of HA releases H^+ . The translocation of A^- is slow and may require complex formation with a cation. The fast translocation of H^+ on the addition of acid will be stoichiometric with the lipid-soluble weak-acid component. Similarly, a fast translocation of H^+ will be catalysed by a lipid-soluble weak base on the addition of OH^- to one side of the membrane.

These findings could explain the results of the present experiments in which the lipid-soluble weak base dibucaine or the fatty acid oleate are added to the proteoliposomes (Figs. 6 and 7). The extent of the fast phase following either acid or alkali addition will depend on the relative amounts of each of the lipid-soluble components in the bilayer. The ability of fatty acids to act as transmembrane cation carriers is also suggested from the results of experiments on Fe^{3+} transport into liposomes (Simpson & Peters, 1987; Simpson *et al.*, 1988) and Ca^{2+} translocation into liposomes and organic solvents (Serhan *et al.*, 1981; Kim & LaBella, 1985). It would appear that fatty acids have a significant ability to cross bilayers as cation-complexed soaps.

The H^+/OH^- permeability of the vesicle membranes can be calculated from the results of the passive flux experiments, according to Deamer & Nichols (1983), using the equation:

$$J_{\text{H}} = P_{\text{H}} A \Delta\text{pH}^+$$

where J_{H} is the proton flux across the membrane in response to an imposed proton concentration gradient of ΔH^+ , P_{H} is the permeability coefficient for net H^+/OH^- movement and A is the surface area of the membrane system. J_{H} can be calculated from the measured rate of pH change in the passive flux experiments, using a knowledge of the volume, V , of the vesicles. In this case we have:

$$J_{\text{pH}} V = P_{\text{H}} A \Delta\text{pH}$$

However, the presence of buffer increases the amount of protons required to cause the increase in pH. To correct for this, it is necessary to know the increase in pH and the amount of buffer present when the value for k ($J_{\text{pH}}/\Delta\text{pH}$) was calculated. In Fig. 4, the internal buffer concentration was 100 mM-Hepes ($\text{p}K_{\text{a}}$ 7.55) and the internal pH change was from 7.29 to 7.51. Under these conditions, essentially all of the OH^- can be assumed to deprotonate the buffer. Therefore the concentration of OH^- required to convert 100 mM-Hepes from pH 7.29 to 7.51 can be calculated from the Henderson-Hasselbalch equation to be 17.2 mM, whereas the change in free OH^- concentration is only 2.1×10^{-8} M. Near the $\text{p}K_{\text{a}}$ of the buffer, the change in pH is proportional to the amount of OH^- added. The correction for buffer capacity, therefore, is simply $\Delta[\text{BH}]/\Delta[\text{H}^+]$. We have finally:

$$P_{\text{H}} = k(\Delta[\text{BH}]/\Delta\text{H}^+)(V/A)$$

With a value for k of 0.0015 s^{-1} (Fig. 5), P_{H} turns out to be $6.1 \times 10^{-4} \text{ cm} \cdot \text{s}^{-1}$. This assumes an average vesicle diameter of 30 nm and an internal buffer (Hepes) concentration of 100 mM. Additional buffering capacity could arise from internally facing phospholipid and protein, and this would slightly raise the final value. This value is in general agreement with previously reported values of P_{H} measured in other systems (Deamer & Bramhall, 1986) and indicates that the presence of oxidase, under non-turnover conditions, does not appreciably alter the bilayer permeability to H^+ . An estimate of the passive permeability of the vesicles to K^+ (P_{K}) can also be made, using the H^+ permeability rate in the presence of FCCP. With FCCP present, we can assume that K^+ counter-ion flow is rate limiting to H^+ movement (Fig. 3). Thus the K^+ flux has the same value as the measured H^+ flux, but at a much greater ion gradient (around 17 mM- K^+ at this pH). A P_{K} value of $7.5 \times 10^{-10} \text{ cm} \cdot \text{s}^{-1}$ is found, which is slightly higher than reported values for the intrinsic bilayer permeability for K^+ , normally around 10^{-10} – $10^{-12} \text{ cm} \cdot \text{s}^{-1}$ (Deamer & Bramhall, 1986). In this calculation, however, no correction has been made for the electrical driving force of the H^+ diffusion potential (around 13 mV) created by the presence of FCCP. This would increase K^+ counterflow above that occurring in the absence of a diffusion potential. The calculated K^+ permeability

Fig. 9. Minimal model for proteoliposome steady state

H^+ removal from the vesicle interior by O_2 reduction and H^+ translocation is catalysed by cytochrome oxidase (J_{ox}). This is balanced by H^+ influx via electrogenic (J_{H}) and electroneutral ($J_{\text{H/K}}$) pathways through the vesicle membrane. The electrogenic entry of K^+ (J_{K}) is balanced by an electroneutral efflux pathway ($J_{\text{H/K}}$).

coefficient will therefore be larger than expected. Nevertheless, the results do illustrate the striking difference in permeability of phospholipid bilayers to H^+ compared with other cations.

Modelling the steady state

With a knowledge of the size, capacitance and membrane permeabilities, what is the simplest model that is sufficient to explain the experimental proteoliposomal ion gradients? A kinetic model has been devised (Fig. 9) which makes the following assumptions. (i) Electrons are transferred (at a rate J_{ox}) from the external cytochrome *c* to the internal face of the enzyme, where one internal proton is consumed per electron in the O_2 reduction reaction. (ii) A proton pump translocates one proton per electron at steady state from the inside to the outside of the vesicle. (iii) The events of (i) and (ii) generate an internal alkalization and a membrane potential (negative inside). These gradients inhibit cytochrome oxidase turnover (respiratory control). (iv) J_{H} is proportional to ΔpH in the absence of $\Delta\psi$ (ohmic response). J_{H} responds to $\Delta\psi$ in a non-ohmic manner (Krishnamoorthy & Hinkle, 1984; O'Shea *et al.*, 1984).

With these assumptions, it can be seen that initiation of turnover results in the electrogenic translocation of H^+ out of the vesicle by cytochrome oxidase. This causes an immediate rise in $\Delta\psi$ and a small alkalization of the vesicle interior. In response to these initial changes, there will be an electrogenic movement of H^+ and K^+ into the vesicle (J_{H} and J_{K}). From the traces shown in Fig. 1, it is apparent that internal alkalization slowly increases during this phase and also that the turnover of the enzyme is slightly inhibited (e.g. see Fig. 1b). Singh & Nicholls (1984) have demonstrated a specific K^+ influx, which is proportional to oxidase turnover, over this period. The enzyme was shown to drive active K^+ uptake even in the presence of a 50-fold excess of Na^+ over K^+ . These authors concluded that some specific K^+ mediator is present in the proteoliposomes, at least in those prepared by sonication. From Fig. 9, it can be seen that the electrogenic entry of K^+ , driven by the membrane potential, would result in the accumulation of KOH inside the vesicle. The rise in ΔpH can be seen from Fig. 1 to be accompanied by a small fall in $\Delta\psi$.

What happens at steady state? Does K^+ entry continue until it reaches Donnan equilibrium? In such a case, a $\Delta\psi$ of around 120 mV under the present conditions, with 100 mM- K^+ in the medium, would require an internal concentration of 10 M-KOH. With the internal buffer concentrations used, this would lead to

far greater changes in ΔpH than are observed. An alternative explanation could be that K^+ reaches a kinetic steady state by the action of endogenous electroneutral exchangers in the membrane, catalysing the movement of K^+ out of the vesicle (Fig. 9). Indeed, as shown in Fig. 1, the steady-state ΔpH level can be varied by the addition of small amounts of nigericin, an electroneutral K^+/H^+ exchanger. We therefore make the additional assumption: (v) K^+ reaches a kinetic steady state rather than a Donnan equilibrium via J_{K} and $J_{\text{H/K}}$ flux pathways. Large K^+ gradients do not form.

From (i) and (ii), each electron passing through the enzyme will translocate two charges across the membrane and remove two internal protons. For a dialysed vesicle of diameter 30 nm containing 100 mM-Hepes at pH 7.5, it can be calculated (Table 1) that each ion crossing the membrane will generate (or collapse) 15.9 mV of membrane potential. Under the same conditions, each proton removed from the vesicle will generate 0.0067 ΔpH . Therefore, for $\Delta\psi$:

$$\text{Rate of formation (mV} \cdot \text{s}^{-1}) = J_{\text{ox.}} \times 2 \times 15.9 \quad (1)$$

$$\text{Rate of decay (mV} \cdot \text{s}^{-1}) = (J_{\text{H}} + J_{\text{K}}) \times 15.9 \quad (2)$$

For ΔpH :

$$\text{Rate of formation (}\Delta\text{pH} \cdot \text{s}^{-1}) = J_{\text{ox.}} \times 2 \times 0.0067 \quad (3)$$

$$\text{Rate of decay (}\Delta\text{pH} \cdot \text{s}^{-1}) = (J_{\text{H}} + J_{\text{H/K}}) \times 0.0067 \quad (4)$$

J_{H} ($\text{H}^+ \cdot \text{s}^{-1}$) and J_{K} ($\text{K}^+ \cdot \text{s}^{-1}$) are probably not simple functions of the electrochemical potentials. The suggested relationships are:

$$J_{\text{H}} = (\Delta\text{pH} \cdot a) + (\Delta\psi^2 \cdot a') \quad (5)$$

$$J_{\text{K}} = (\Delta\psi^2 \cdot b) \text{ (minus valinomycin)} \quad (6)$$

$$J_{\text{K}} = (\Delta\psi^2 \cdot b) + (\Delta\psi \cdot b') \text{ (plus valinomycin)} \quad (7)$$

where the constant a is in units $\text{H}^+ \cdot \text{s}^{-1} \cdot \Delta\text{pH}^{-1}$, a' is in units $\text{H}^+ \cdot \text{s}^{-1} \cdot \Delta\psi^{-2}$, b is in units $\text{K}^+ \cdot \text{s}^{-1} \cdot \Delta\psi^{-2}$ and b' is in units $\text{K}^+ \cdot \text{s}^{-1} \cdot \Delta\psi^{-1}$.

Using an exponential term instead of the square term in eqns. (5) and (6) has little effect on the final model. In the presence of valinomycin, the J_{K} component may be ohmic in character, depending on the values of b and b' .

The electroneutral movement of potassium is modelled by:

$$J_{\text{H/K}} = \Delta\text{pH} \cdot c \text{ (minus nigericin)} \quad (8)$$

$$J_{\text{H/K}} = \Delta\text{pH} \cdot (c + c') \text{ (plus nigericin)} \quad (9)$$

where c and c' are constants with units $\text{H}^+ \cdot \text{s}^{-1} \cdot \Delta\text{pH}^{-1}$.

The activity of the enzyme is given by:

$$J_{\text{ox.}} (\text{e}^{-1} \cdot \text{s}^{-1}) = J_{\text{min.}} + J_{\text{unc.}} [k/(k + \Delta\text{pH})] [k'/(k' + \Delta\psi^2)] \quad (10)$$

where $J_{\text{min.}}$ represents a minimal coupled flux (in the absence of ionophores) and $J_{\text{min.}} + J_{\text{unc.}}$ is the maximum uncoupled flux (in the presence of ionophores). k and k' then represent the values at which $J_{\text{unc.}}$ is 50% inhibited by ΔpH and $\Delta\psi^2$ respectively. This is a phenomenological equation, but not an arbitrary one. It is assumed that (i) control is kinetic, not thermodynamic ($J_{\text{min.}}$ would decline to zero as the thermodynamic reversal $\Delta\mu\text{H}^+$ is approached); (ii) either ΔpH or $\Delta\psi$ can control the enzyme (neither nigericin nor valinomycin give full release of respiratory control); and (iii) control may be non-ohmic.

To model the changes in $\Delta\psi$ and ΔpH with time and ionophore additions, the above equations (1–10) were converted into differential equations and numerically integrated as described in the Materials and methods section. Fig. 10 shows a simulated experiment and can be compared with the experimental traces shown in Figs. 1 and 4. In agreement with the results, the initial translocation of protons causes a sharp rise in $\Delta\psi$ (Fig. 10a) and

Fig. 10. Simulation of respiration-dependent ΔpH and $\Delta\psi$ changes occurring in cytochrome oxidase proteoliposomes

The effects of different rates of K^+/H^+ exchange on the steady-state levels of $\Delta\psi$ (a) and ΔpH (b) have been compared. For the initial approach to steady state (left-hand curves), the model was as described in the text with the following numerical values for the constants (units given in text): $a = 59.7$, $a' = 0.012$, $b = 0.0008$, $c = 15$, $J_{\text{min.}} = 10$, $J_{\text{unc.}} = 140$, $k = 0.1$, $k' = 6000$. After steady state has been reached, the effect of increasing or decreasing the K^+/H^+ exchange activity is shown by varying the value of c in the model from 0 to 1500 as shown in the traces. Note the different time scales for the left-hand and right-hand curves.

a smaller rise in ΔpH (Fig. 10b). The increased rate of K^+ uptake at higher membrane potentials then starts to decrease $\Delta\psi$. As the potential decreases, the rate of proton influx decreases, resulting in a concomitant increase in ΔpH . The gradients and ion fluxes then approach a steady state where:

$$J_{\text{ox.}} \times 2 = J_{\text{H}} + J_{\text{H/K}}$$

and

$$J_{\text{K}} = J_{\text{H/K}}$$

In the presence of high levels of valinomycin and nigericin, the simulated values of $\Delta\psi$ and ΔpH are lowered to zero (as seen experimentally in Fig. 1) with resulting increases in enzyme turnover.

Fig. 10 also shows that a model with $J_{\text{H/K}} = 0$ is unable to explain the experimental data. Simulated changes in $\Delta\psi$ and ΔpH are shown with varying K^+/H^+ exchange activity. (Decreasing the intrinsic K^+/H^+ exchange rate is difficult to achieve experimentally and shows one advantage of using a model.) As expected, increasing the K^+/H^+ activity increases $\Delta\psi$ and decreases ΔpH such that at saturating levels the protonmotive force consists solely of $\Delta\psi$ (see the effect of added nigericin in Fig. 1). Decreasing the K^+/H^+ activity in the vesicles, however, has the opposite effect such that, in the absence of any electroneutral exchange, the membrane potential is totally converted into a pH gradient. The model clearly shows that the need for electroneutral exchange activity in maintaining a steady-state membrane potential is as important in proteoliposomes as it is in mitochondria (Mitchell, 1968). The simulations support the argument for the presence of endogenous electroneutral exchangers in the vesicle membranes.

H⁺ permeability under turnover conditions

Under steady-state turnover conditions, the net flux of all ions into and out of the proteoliposome must be zero. It is therefore possible from the present results to put some values to the individual flux pathways shown in Fig. 9. An oxidase turnover of around 30 electron equivalents $\cdot s^{-1}$, under respiratory control and in the presence of valinomycin (Fig. 4), would remove 60 H⁺ equivalents $\cdot s^{-1}$ from the vesicle interior. With 16.6 mm-phospholipid per μM of aa_3 (see the Materials and methods section), and a trapped volume of 0.5 litres per mole of phospholipid (Wrigglesworth, 1985), the rate of H⁺ removal from the vesicle interior by the enzyme would then be 7.2 mm-H⁺ $\cdot s^{-1}$. This has to be matched by an equivalent entry of H⁺ via electrogenic (J_H) and electroneutral ($J_{H/K}$) pathways. At steady state, in the presence of valinomycin to maintain a chemical rather than an electrochemical driving force, the system appears to support a ΔpH of around 0.3. Using the above value of 7.2 mm $\cdot s^{-1}$ for J_H , the apparent P_H under turnover conditions turns out to be 0.14 cm $\cdot s^{-1}$. This is much higher than the equivalent value found under passive conditions. A decrease of stoichiometry of proton pumping by the oxidase under steady-state conditions, as suggested by Murphy & Brand (1988a,b), would decrease H⁺ removal from the interior but would not affect the scalar consumption of protons. J_H would only be lowered by at most a factor of 2, insufficient to explain the calculated difference. At first sight, therefore, it would appear that oxidase turnover increases the intrinsic permeability of the vesicle to protons.

Could there be an artifactual basis to the above calculations? Some H⁺ flux will be supported by the electroneutral pathway ($J_{H/K}$) and this flux is not included in the permeability calculations. Nevertheless, there is no reason to believe that electroneutral exchange would be any different under active rather than passive conditions and therefore this could not be responsible for the disparity. A more likely explanation is that vesicles incorporating oxidase could be intrinsically more permeable to H⁺ than vesicles without incorporated enzyme. A heterogeneous mixture of such vesicles would then lead to different calculated results according to whether the pH gradients were imposed passively (to all vesicles) or actively (only to those incorporating enzyme). This argument is supported by the results shown in Fig. 2. The passive decay of the pH gradient generated by oxidase turnover on anaerobiosis must reflect events in oxidase-containing vesicles only. It can be seen that the decay of the pH gradient generated by oxidase turnover is faster than that seen when a pH gradient is imposed on all of the vesicles by the external addition of alkali or acid. A first-order rate constant of $3.9 \times 10^{-3} s^{-1}$ characterizes the decay of the oxidase-generated gradient. This is approximately 2 times faster than the rate in Fig. 5, which comes from the decay of a pH gradient imposed on all vesicles. This is still much lower than the value calculated from oxidase turnover. However, two further effects of vesicle heterogeneity must be considered. The first is that the pH gradient generated under turnover conditions would be underestimated, since calibration of the fluorescence changes is done by addition of external acid or alkali to all vesicles in the sample. For this to affect the calculation of P_H under turnover conditions by a factor of 10^2 requires oxidase-containing vesicles to generate a ΔpH of around 2 pH units. Since the passive calibration indicates a ΔpH of around 0.3, then this would imply that only 15% of the trapped volume represents oxidase-containing vesicles. Such a high degree of heterogeneity is at variance with previous measurements on oxidase vesicles prepared by dialysis (Casey *et al.*, 1984; Sarti *et al.*, 1989). In addition, measurements on the present preparations have indicated an association of pyranine and oxidase with

all size vesicles in both dialysed and sonicated preparations (Wrigglesworth *et al.*, 1987; Cooper & Nicholls, 1990). A third effect that could contribute to an overestimation of P_H under turnover conditions is heterogeneity of respiratory control. A few vesicles with high control (low turnover) could be mixed with many vesicles with low control (high turnover). This would result in the value used for J_H in the calculation being too high. Again, however, to explain the calculated difference, J_H would have to be lowered by a factor of 100, implying respiratory control ratios of several hundred in the coupled vesicles.

A combination of the above effects could contribute to the apparent overestimation of P_H under turnover conditions, but it is difficult to explain the full effect in this manner. An alternative explanation for the apparently higher P_H under turnover conditions may be that, during steady state, cytochrome oxidase itself provides a channel for H⁺ backflow. As discussed by Proteau *et al.* (1983), an apparent decrease in H⁺/e⁻ stoichiometry with decreased turnover rate could result from an intrinsic leak in the system when the enzyme was active. Such a leak would also explain the apparent decrease in the H⁺/e⁻ ratio seen in oxygen pulse and cytochrome *c*²⁺ pulse experiments with increasing enzyme turnovers (Casey *et al.*, 1979; Solioz *et al.*, 1982). Intrinsic uncoupling of cytochrome *c* oxidase in proteoliposomes during turnover has also been proposed by Maison-Peteri & Malmstrom (1989), although the effect was only said to be significant at pH values below 7.

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