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TURNOVER AND VECTORIAL PROPERTIES OF CYTOCHROME *c* OXIDASE IN RECONSTITUTED VESICLES

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Summary

1. Proteoliposomes containing cytochrome *c* oxidase and phospholipid have been made by sonication and by the cholate dialysis procedure. In both methods of preparation, only about 50% of the enzyme molecules are oriented in the membrane with their cytochrome *c* reaction sites exposed to the outside of the vesicle.

2. The activity of cytochrome *c* oxidase in the reconstituted vesicles is not increased by incubation in 1% Tween 80. Experiments on reconstituted vesicles containing internal (entrapped) cytochrome *c* indicate that turnover of enzyme oxidising entrapped cytochrome *c* in the presence of *N,N,N',N'*-tetramethyl-*p*-phenylenediamine or 2,3,5,6-tetramethyl-*p*-phenylenediamine is at a very much lower rate than enzyme oxidising external ferrocytochrome *c*.

3. Oxidation of ascorbate by externally added cytochrome *c* results in an electrogenic production of OH^- inside the vesicles, which can be monitored using entrapped phenol red. Polylysine inhibits, but does not abolish, the internal alkalinity change in reconstituted vesicles oxidising internal (entrapped) cytochrome *c* using externally added ascorbate plus *N,N,N',N'*-tetramethyl-*p*-phenylenediamine. When 2,3,5,6-tetramethyl-*p*-phenylenediamine is used as the permeable redox mediator, an increase in internal acidity can be monitored under the same conditions.

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Abbreviations: TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; DAD, 2,3,5,6-tetramethyl-*p*-phenylenediamine; FCCP, *p*-trifluoromethoxycarbonylcyanide phenylhydrazone.

Introduction

Reconstituted phospholipid vesicles containing cytochrome *c* oxidase have been used in many ways to study the vectorial properties of the enzyme in isolation from other components of the mitochondrial respiratory chain [1—4]. Under suitable conditions, the reconstituted system can be shown to create a membrane potential [2,5] and undergo respiration-dependent exchange of protons for K^+ across the membrane [1]. Distinct energy-dependent changes in the redox and spectral properties of cytochrome *c* oxidase have also been demonstrated in reconstituted vesicles [23]. When incorporated into a phospholipid membrane together with mitochondrial ATPase, the oxidase is also able to couple the free energy of oxygen reduction to ATP synthesis [6].

Reconstituted vesicles have usually been prepared by one of two general procedures. Either a suitable mixture of phospholipids has been co-sonicated with purified cytochrome oxidase [7] or the two have been mixed together in the presence of detergent and the detergent then diluted out or removed by dialysis [8]. In general, both preparation methods have given similar results in terms of respiratory control and respiration-dependent ion movements. However, there are conflicting reports with regard to the orientation of the enzyme in the vesicle membrane. Thus Carroll and Racker [9] report that cytochrome *c* oxidase, when the only protein present, assembles in the membrane in the mitochondrial orientation, whilst Wrigglesworth [10] and Nicholls [11] provide evidence for a more 'scrambled' orientation with about 50% of the enzyme having its cytochrome *c* reaction site exposed to the outside. These differences may arise from the different methods of preparation or may result from the different methods used to assess the orientation of the enzyme in the membrane.

In this paper we describe experiments on reconstituted vesicles prepared both by sonication and by detergent dialysis. The use of suitable combinations of membrane permeable and impermeable reductants has permitted the measurement of vectorial reactions of the enzyme in either orientation across the vesicle membrane. It has been found that for both methods of preparation, only about half of the cytochrome aa_3 in the vesicles can be reduced by ascorbate plus cytochrome *c* under anaerobic conditions. However, it has also been found that only those enzyme molecules having their cytochrome *c* reaction site exposed to the outside of the vesicle are capable of turnover rates comparable to those of the enzyme in free solution. A preliminary report has been made of some of the work described [10].

Materials and Methods

Keilin-Hartree type beef heart submitochondrial particles were prepared according to Nicholls [12]. Cytochrome *c* oxidase was prepared from the particles essentially as described by Kuboyama et al. [13] with Tween 80 substituting for Emasol. The final preparations of the enzyme had a haem aa_3 : protein ratio between 4 and 5 $\mu mol \cdot g^{-1}$ and maximal turnovers (electrons/aa_3) of 100—200 s^{-1} in the presence of 0.05% asolectin, 50 mM potassium phosphate, pH 7, with ascorbate and horse heart cytochrome *c* (Sigma, type VI). Spectra

were obtained with an Aminco DW-2 spectrophotometer. Phosphatidylcholine was purified from egg yolk according to Singleton et al. [14]. Phosphatidylethanolamine (egg yolk) and cardiolipin (ox heart) were purchased from Lipid Products, Nutfield Nurseries, South Nutfield, Surrey, U.K. Asolectin was supplied by Associated Concentrated, Long Island, NY, U.S.A. Nigericin was a gift from Dr. J. Berger, Hoffman-La Roche Inc., NJ, U.S.A. Other reagents and methods were of the usual laboratory kind.

Reconstitution by sonication was performed essentially according to Racker [7]. The normal procedure was to disperse the dry phospholipids (asolectin or a mixture of phosphatidylcholine, phosphatidylethanolamine and cardiolipin in the proportions 2 : 2 : 1 (by weight), respectively) in 50 mM potassium phosphate (pH 7.4) by rapid shaking. Cytochrome *c* oxidase was then added to a final phospholipid : protein weight ratio of approximately 20. The mixture (usually 2 ml) was well shaken, placed in a container surrounded by ice and sonicated to clarify (usually 1—2 min) with a Bronwill sonifier at maximum intensity. Short periods of sonication were used to avoid overheating. The mixture was then centrifuged at 50 000 × *g* for 15 min and any pellet discarded. The phospholipid : protein ratio was not significantly altered by the centrifugation procedure but small amounts of the mixture (less than 10%) together with tungsten particles from the sonicator probe were removed. Reconstituted oxidase vesicles containing entrapped phenol red or phenol red plus cytochrome *c* were prepared by sonicating the phospholipid/enzyme mixture in the presence of 5 mM phenol red or 5 mM phenol red plus 200 μM cytochrome *c*. The internal buffer concentration of these vesicles was reduced by using 5 mM potassium phosphate plus 45 mM KCl. After sonication, the mixture was passed down a column of Sephadex G-75 equilibrated with 50 mM potassium phosphate (pH 7.4) to separate vesicles from excess cytochrome *c* and phenol red. Calibration of internal pH changes was done by monitoring spectral changes at 557 nm in vesicles suspended in 50 mM potassium phosphate buffer at different pH values in the presence of valinomycin plus nigericin but in the absence of any reductants. Reconstitution by cholate dialysis was according to Racker [8].

Results

Characteristics of the reconstituted vesicles

Vesicles prepared by the cholate dialysis method have already been well characterised [9,15]. Vesicles prepared by sonication do not differ qualitatively from cholate vesicles in their properties. Thus, electron microscopy of reconstituted sonicated vesicles showed that the preparation comprises single-layered vesicles, heterogeneous in size with the majority ranging in diameter between 20 nm and 50 nm. When the vesicle preparation is passed down a column of Sepharose 2B (Pharmacia, Uppsala, Sweden) the elution pattern (Fig. 1) indicates association of the oxidase with phospholipid, but with the enzyme tending to associate perferentially with the smaller vesicles. 'Respiratory control' ratios (the ratio of the respiration rate in the presence of valinomycin plus *p*-trifluoromethoxycarbonylcyanide phenylhydrazone (FCCP) to that in the absence of ionophores) were higher in the smaller vesicles as

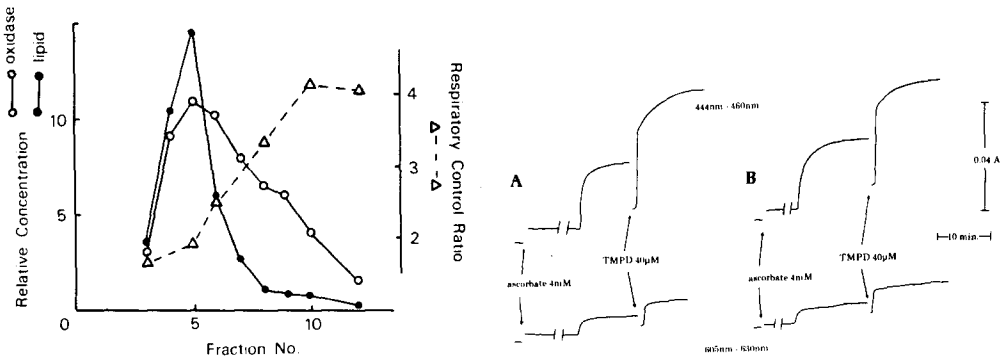

Fig. 1. Elution pattern of sonicated vesicles on a column of Sepharose 2B. Vesicles were prepared as described in the text and 2 ml loaded on to a column (2 cm^2 X 15 cm) of Sepharose 2B preequilibrated with 50 mM potassium phosphate, pH 7. The relative lipid content of the fractions (2 ml) was taken to be proportional to the absorbance of the sample at 460 nm. Measurement of organic phosphate on several vesicle samples showed this assumption to be valid. The relative cytochrome *c* oxidase content was derived from measurements of respiratory rates of the individual fractions according to Wrigglesworth and Nicholls [28] and from the 444—460 nm absorption of the fractions after dithionite reduction. Both methods of oxidase estimation gave similar results. Respiratory control ratios were measured as described in the text.

Fig. 2. Degree of reduction of cytochrome *c* oxidase in reconstituted vesicles in the presence of externally added cytochrome *c* and TMPD. Reconstituted vesicles were suspended to a final aa_3 concentration of 0.9 μM in 50 mM potassium phosphate (pH 7) and 1.6 μM cytochrome *c*, and the absorption differences 444—460 nm and 605—630 nm monitored. TMPD was added to the anaerobic sample after approximately 10 min. A. Vesicles prepared by sonication. B. Vesicles prepared by the cholate dialysis method.

reported for vesicles prepared by cholate dialysis by Carroll and Racker [9]. The response of cytochrome *c* oxidase in the reconstituted vesicles to valinomycin plus FCCP when oxidising externally added cytochrome *c* was similar to that reported previously [10] involving respiratory control ratios between 3 and 5. Vesicles prepared by cholate dialysis gave similar control ratios. No consistent differences were noted between reconstituted vesicles prepared using cardiolipin plus egg yolk phospholipids and those prepared using asolectin.

Orientation of cytochrome c oxidase in the vesicle membrane

In the presence of externally added ascorbate and cytochrome *c* only 50—60% of the oxidase molecules are fully reduced when the sample becomes anaerobic (Fig. 2). Similar results are obtained whether the vesicles are prepared by sonication (Fig. 2A) or by cholate dialysis (Fig. 2B). Reduction of the remaining oxidase molecules under anaerobic conditions requires the addition of a membrane-permeable reductant such as *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD). It would appear that, for both methods of preparation, approximately half of the oxidase molecules are orientated so as to expose to their cytochrome *c* reaction site to the external medium. The remainder are inaccessible for direct reaction with externally added cytochrome *c*.

An alternative method of estimating orientation of the enzyme was used by Carroll and Racker [9] in which the uncoupled rate of respiration driven by external ferrocytochrome *c* is compared to the activity obtained after disrup-

tion of the liposomes by a detergent which does not inhibit enzyme activity. Fig. 3 shows the effect of 1% Tween 80 on vesicles reconstituted by the sonication method. As with the cholate dialysis vesicles of Carroll and Racker [9], the activity of the enzyme in reconstituted sonicated vesicles, in the presence of uncouplers, is not increased by the addition of 1% Tween 80 although, as would be expected, the detergent abolishes the effects of the ionophores. The turnover of purified cytochrome *c* oxidase, in the presence of asolectin, is also not affected by the detergent (results not shown). Comparison of the turnovers of the vesicle preparations in the presence of Tween 80 and of the purified enzyme in the presence of the same amount of phospholipid and detergent shows that an equivalent activity to that of the purified enzyme is shown by those cytochrome *c* oxidase molecules with cytochrome *c* reaction sites exposed to the external medium. From the previous experiments (Fig. 2), these molecules constitute only 50—60% of the total cytochrome *c* oxidase in the preparation. However, it should be noted that the maximal oxidase activity at 30°C and pH 7.4 (300 s^{-1}) is usually down by comparison with mitochondrial enzyme (450—500 s^{-1}) [16].

Turnover of enzyme oxidising internal (entrapped) cytochrome c

Reconstituted vesicles sonicated in the presence of cytochrome *c* and then passed down a column to remove excess cytochrome *c* (see Materials and Methods) retain approximately 70% cytochrome *c* which is non-reducible by externally added ascorbate (Fig. 4, inset). Turnover of the enzyme in these preparations, using externally added cytochrome *c* and ascorbate plus TMPD, can be inhibited approximately 90% by externally added polylysine (Fig. 4) indicating that turnover of enzyme oxidising entrapped cytochrome *c* is very much slower than that of enzyme oxidising external ferrocytochrome *c*. The final respiratory rate is only slightly greater than that which would be expected from ascorbate plus TMPD in the absence of cytochrome *c*. A similar results is obtained when diaminodurene (DAD) is used as a redox mediator instead of TMPD (Fig. 5). This compound can permeate membranes in the protonated

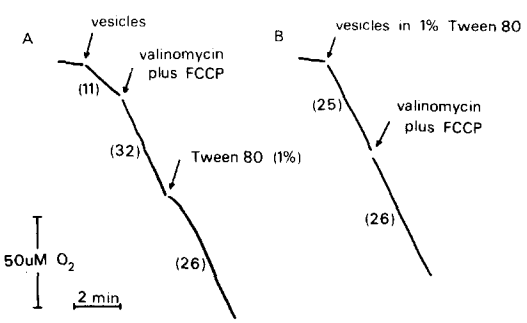

Fig. 3. Effect of Tween 80 on the turnover of cytochrome *c* oxidase in reconstituted vesicles. Reconstituted vesicles prepared by sonication were suspended to a final aa_3 concentration of 16 nM in 3 ml of 50 mM potassium phosphate, pH 7, containing cytochrome *c* (33 μM), potassium ascorbate (3.3 mM) and TMPD (33 μM). Oxygen uptake at 30°C was monitored by an oxygen electrode. Figures in brackets indicate μM $O_2 \cdot min^{-1}$ after corrections for autooxidation and dilutions. In trace (B), the vesicles were preincubated in 1% Tween 80 for 10 min before addition. Ionophore concentrations were valinomycin (0.17 μg · ml^{-1}) and FCCP (1.7 μM).

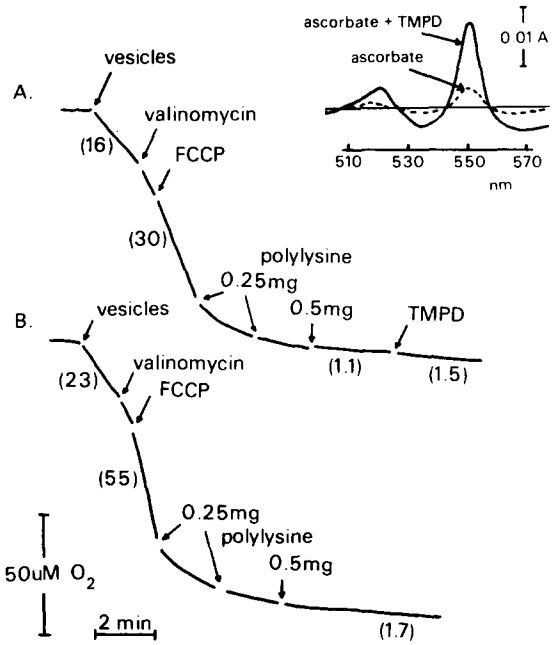

Fig. 4. Effect of polylysine in the respiration rate of reconstituted vesicles containing entrapped cytochrome *c*. Vesicles prepared by sonication, containing entrapped cytochrome *c*, were added to a final aa_3 concentration of 100 nM to 3 ml of 10 mM potassium phosphate (pH 7) containing externally added cytochrome *c* (66 μM) and potassium ascorbate (3.3 mM). In trace (A), the addition of TMPD was to a final concentration of 33 μM. Further addition of TMPD to 280 μM gave the same result. In trace (B), TMPD (33 μM) was added initially. Oxygen uptake was monitored at 23°C. Ionophore concentrations as in Fig. 3. Inset. Anaerobic difference spectra of vesicles (0.8 μM aa_3) in 10 mM potassium phosphate, pH 7, plus potassium ascorbate (3.3 mM) and potassium ascorbate plus TMPD (33 μM).

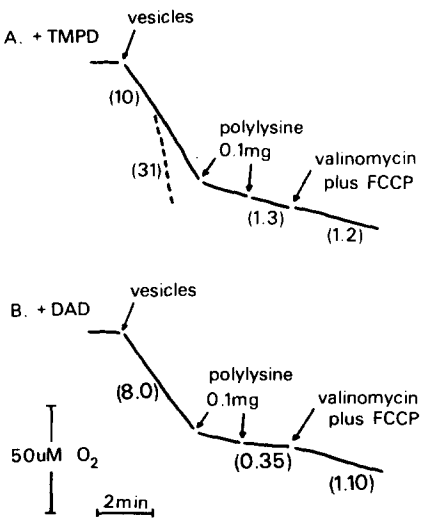

Fig. 5. Comparison of TMPD- and DAD-supported respiration in reconstituted vesicles containing entrapped cytochrome *c*. Vesicles prepared by sonication, containing entrapped cytochrome *c*, were added to a final aa_3 concentration of 50 nM to 3 ml potassium phosphate (50 mM) (pH 7) containing externally added cytochrome *c* (33 μM), potassium ascorbate (3.3 mM) and either (A) TMPD (33 μM) or (B) DAD (33 μM). Oxygen uptake was monitored at 23°C. - - - - - -, the respiration rate on the addition of valinomycin (0.17 $\mu g \cdot ml^{-1}$) plus FCCP (1.7 μM) before polylysine inhibition.

(reduced) form (Wrigglesworth, J.M., unpublished observations) and releases protons when oxidised [17]. Unlike TMPD, DAD-supported respiration can still be stimulated significantly by ionophores after polylysine inhibition (Fig. 5).

The characteristic spectrum of $TMPD^+$ (Wursters blue) can be seen in preparations of vesicles respiring on internal cytochrome *c* with ascorbate and higher concentrations of TMPD in the presence of polylysine (Fig. 6). The steady-state concentration of $TMPD^+$ can be reduced by addition of FCCP but surprisingly neither valinomycin or nigericin, added separately or in combination, have any effect. A slightly increased steady-state level of reduced cytochrome *c* accompanies the drop in $TMPD^+$ steady-state concentration suggesting that the specific effect of FCCP may be to release $TMPD^+$ from negative binding sites within the vesicle in a manner similar to the specific effect of uncouplers on bound 8-anilinonaphthalenesulphonic acid [24]. The subsequent efflux of 'free' $TMPD^+$ could increase the steady-state level of reduced cytochrome *c* inside the vesicles. A similar effect can be seen in Keilin-Hartree particles when high (greater than 100 μM) concentrations of TMPD are used (Miller, M., Hansen, F.B., Petersen, L.Chr. and Nicholls, P., unpublished results).

Internal pH and oxidase turnover

Changes in internal pH of the vesicles can be monitored by following changes in entrapped phenol red absorption [8,10]. It can be seen in Fig. 7 that reconstituted vesicles prepared by sonication have limited permeability to both K^+ and H^+. The addition of polylysine (of average molecular weight 90 000) to the

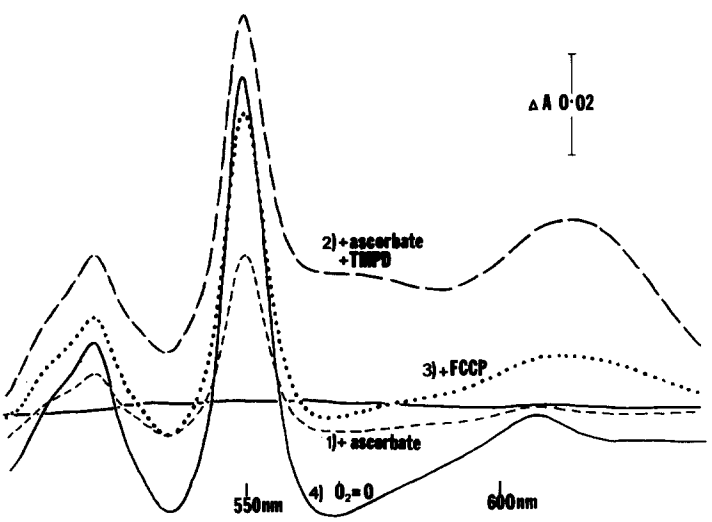

Fig. 6. Respiration-dependent spectral change in reconstituted vesicles containing internal cytochrome *c* and respiring on ascorbate plus TMPD. Reconstituted vesicles of ctyochrome *c* oxidase containing internal cytochrome *c* were suspended in 50 mM potassium phosphate (pH 7.4) containing 90 $\mu g \cdot ml^{-1}$ polylysine (average molecular weight 90 000). Sodium ascorbate (10 mM), TMPD (150 μM) and FCCP (1 μM) were added successively to the sample cuvette and the difference spectrum recorded after each addition. The final anaerobic difference spectrum was recorded after 400 s. Spectrum 2 was unaltered by the addition of valinomycin plus nigericin (1 $\mu g \cdot ml^{-1}$ each).

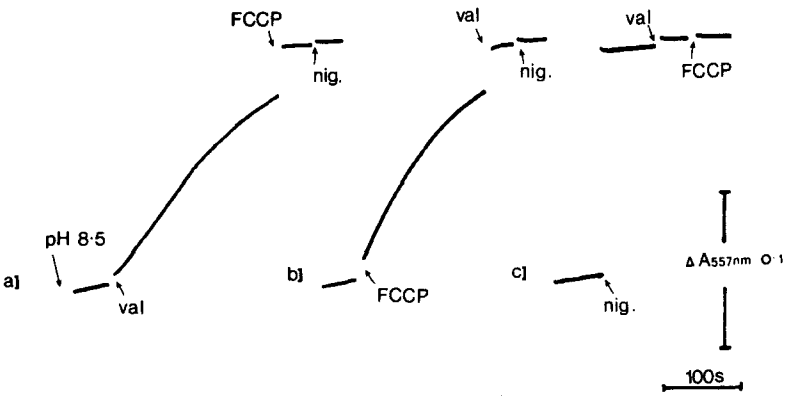

Fig. 7. Permeability of reconstituted vesicles to H^+ and K^+. Reconstituted vesicles of cytochrome *c* oxidase at pH 7.4 containing internal phenol red were suspended at 20°C in 50 mM potassium phosphate (pH 8.5) and changes in phenol red absorption at 557 nm were monitored with time. Ionophore concentrations were valinomycin (val) 0.17 $\mu g \cdot ml^{-1}$, nigericin (nig.) 0.17 $\mu g \cdot ml^{-1}$ and FCCP 1.7 μM.

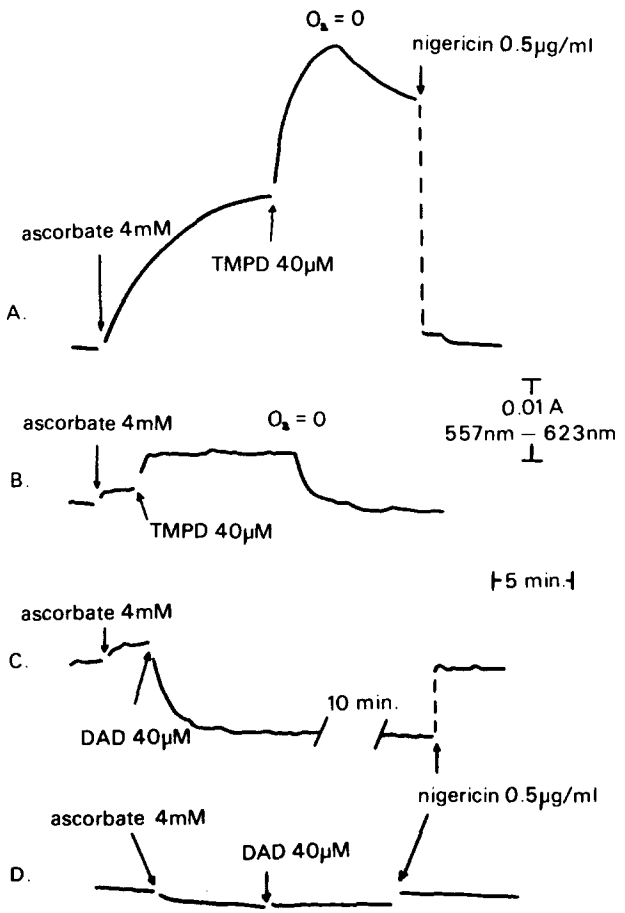

Fig. 8. Effect of polylysine on respiration-dependence pH changes in reconstituted vesicles. Reconstituted vesicles of cytochrome *c* oxidase prepared by sonication and containing entrapped cytochrome *c* and phenol red were suspended in 2.5 ml potassium phosphate (50 mM) (pH 7.2) to a final aa_3 concentration of 0.5 μM. Valinomycin (0.2 $\mu g \cdot ml^{-1}$) was added and changes in phenol red absorption (557—623 nm) were monitored at 23°C after the addition of ascorbate. A As described; B plus 0.3 mg polylysine (average molecular weight 90 000); C as B; D as C except that vesicles prepared without cytochrome *c* oxidase were used.

vesicles at a concentration of 33 $\mu g \cdot ml^{-1}$ had no significant effect on these traces. It has previously been shown that internal alkalinization occurs when the enzyme in the vesicles oxidises external ferrocytochrome *c* [10,18]. Polylysine (0.12 $mg \cdot ml^{-1}$) inhibits but does not abolish the internal alkalinity changes with ascorbate alone or in the presence of TMPD (Fig. 8). The internal alkalinity changes became less as the polylysine ion concentration was increased but in no case was an increase in internal acidity detected under these conditions; such experiments, carried out with sonicated vesicles containing entrapped cytochrome *c*, were done both in the presence and absence of valinomycin with identical results with the exception that valinomycin was found to enhance the initial rate of alkalinity change. That the vesicles were still relatively impermeable to H^+ when polylysine was present is shown if DAD is used as the permeable redox mediator instead of TMPD (Fig. 8c). The addition of DAD to polylysine-inhibited vesicles results in an internal acidity increase. However, the final steady-state respiration rate obtained with proteoliposomes oxidising internal ferrocytochrome *c* in the presence of DAD was extremely slow. The subsequent acidity increase was very small and the preparation did not go anaerobic in the open spectrophotometer cuvette. This is consistent with the respiratory control results shown in Fig. 5b where, in the absence of uncoupler, the respiratory rate of vesicles with entrapped cytochrome *c* in the presence of polylysine is very low when DAD is used as a redox mediator.

Discussion

The proportion of enzyme molecules accessible to externally added cytochrome *c* in previous reconstituted preparations has been in doubt. Calculations based on comparisons of the activity shown by the reconstituted system with the activity of the same preparations in the presence of detergent, or of the purified enzyme in free solution, have been complicated by detergent inhibition and lipid activation effects on the enzyme. The results of the present experiments suggest that there are two populations of cytochrome *c* oxidase molecules in the reconstituted vesicles, whether the vesicles are prepared by sonication or by detergent dialysis. One population can oxidise external ferrocytochrome *c* at a higher turnover rate whilst the other is unable to oxidise externally added ferrocytochrome *c* and also has a low turnover rate when oxidising internal (entrapped) ferrocytochrome *c*. When the vesicles are disrupted by detergent, the activity does not markedly increase. Either all the oxidase molecules are now partially inhibited or the second population of oxidase molecules is still not functionally competent towards added ferrocytochrome *c*. Turnover of this population of molecules always seems to require the presence of a lipid-soluble mediator such as TMPD or DAD. Thus, although cytochrome *c* oxidase incorporates into the membrane of phospholipid vesicles in a random orientation, protein-phospholipid interactions impose a functional asymmetry. Kuboyama et al. [13] have shown that the functional unit of the enzyme, isolated by the method used in the present experiments, is a dimer, i.e. comprises two active oxidase molecules. It may be that it is not possible to incorporate this functional unit into a phospholipid membrane or a phospholipid/

detergent micelle with both aa_3 units having their cytochrome *c* reaction sites exposed to an aqueous phase. Those aa_3 units facing inwards appear to have accessibility problems towards ferrocytochrome *c*. This may go some way towards explaining the low turnover rates and *P/O* ratios found when ATP synthesis is driven by oxidation of internal (entrapped) ferrocytochrome *c* in reconstituted vesicles containing cytochrome *c* oxidase and ATPase [6].

The present results support the idea that respiratory control is imposed on the enzyme by an electrogenic production of OH^- within the vesicles when respiring on external cytochrome *c*. Turnover of enzyme 'facing inwards' is not affected by ionophores when TMPD is used as a lipid-soluble mediator presumably because the (slow) rate of electron transfer across the membrane equals the efflux rate of $TMPD^+$ and sufficient membrane potential is not generated. However, when DAD is used as a reductant the turnover of 'inwards facing' oxidase can be stimulated by ionophores. Both the influx of reduced DAD and the efflux of oxidised DAD is electroneutral. In the steady state this would create a membrane potential since production of H^+ occurs on one side of the membrane when the molecule is oxidised and the transfer of the electrons back across the membrane is catalysed by the oxidase. These processes would appear to occur at a sufficient rate in the reconstituted vesicles to impose respiratory control even though this rate is less than 5% of the maximum rate of the 'outward facing' oxidase.

The most striking vectorial property of cytochrome *c* oxidase as its ability to generate a membrane potential when incorporated into a bilayer. Direct measurements across planar phospholipid membranes incorporating cytochrome *c* oxidase show that the enzyme can generate an electric potential when oxidising reduced cytochrome *c* added to one side of the membrane [19]. When oxidising cytochrome *c* added to the other side of the membrane, the polarity of the potential is reversed. Vectorial electron transfer across the membrane from reductant on one side to an oxygen reaction site on the other side has been proposed as one of the simplest explanations for membrane potential generation [20]. However, Wikström and coworkers has presented evidence that the oxidase in mitochondria [21,26] and in liposomes [27] may catalyse electrogenic proton transport across the membrane with a $2\ H^+/2\ e^-$ stoichiometry. In this case, translocated protons are still detected on the cytochrome *c* side of the membrane even when electron donors are used as reductants for the cytochrome *c*. This has been demonstrated in mitochondria oxidising ferrocyanide [21], or TMPD [25], in the presence of antimycin A although the interpretation of the results has been challenged by Moyle and Mitchell [22]. The original purpose of the pH experiments described in the present work was to look for pH changes in the vesicles dependent on the turnover of inwards facing oxidase. The experiments indeed show that turnover of 'inwards facing' oxidase does not result in internal H^+ production unless a reductant such as DAD, which releases H^+, is used. This could be interpreted as an indication that cytochrome *c* oxidase does not act as a proton pump but simply catalyses electron transfer across the membrane. However, the turnover studies indicate that this interpretation must be made with caution. 'Inward facing' oxidase does not appear to be readily accessible to entrapped ferrocytochrome *c* and its turnover is inhibited. Lack of internal H^+ production from the proton-pumping activity

of 'internal facing' oxidase may be a result of slow turnover rates rather than any intrinsic inability of the oxidase to act as a proton pump.

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