Biochimica et Biophysica Acta, 765 (1984) 30–37
Elsevier

BBA 41470

A QUANTITATIVE CHARACTERISATION OF H^+ TRANSLOCATION BY CYTOCHROME *c* OXIDASE VESICLES

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(Received October 26th, 1983)

Key words: Cytochrome c oxidase; Proton translocation; Vesicle reconstitution; Decay kinetics

A quantitative analysis of H^+ extrusion by reconstituted cytochrome *c* oxidase vesicles is presented with particular regard to the decay kinetics of the extruded proton pulse and to the structural heterogeneity of the vesicle preparation. The decay of the extruded H^+ pulse under conditions typical of those used for its measurement is much slower than expected from the passive proton permeability of the vesicle membranes. It is shown that this apparent anomaly results from insufficient transmembrane charge equilibration via valinomycin and K^+ during oxidase turnover. This situation can be remedied by increasing the valinomycin concentration or by replacing this counterion system with 1 mM tetraphenylphosphonium. Under these latter conditions, the decay kinetics can be described as the sum of two exponential terms. To facilitate interpretation of the proton pump decay kinetics, a structural analysis of the oxidase vesicle preparation is presented. The bulk of the reconstituted vesicles (i.e., those representing approx. 80% of the total oxidase and lipid) are 30–62 nm in diameter. At least 70% of the reconstituted oxidase molecules are contained individually in separate vesicles, indicating that the enzyme monomer is competent in H^+ translocation.

Introduction

Whilst the first demonstrations of an H^+-pumping activity for cytochrome *c* oxidase were performed using mitochondria [1,2] or submitochondrial particles [2], important supportive evidence has come from studies with reconstituted cytochrome *c* oxidase vesicles (e.g. Refs. 2–6). These have shown that when ferrocytochrome *c* is oxidised by oxidase vesicles, there is an extravesicular acidification consistent with maximally 1 H^+ being extruded per cytochrome *c* molecule oxidised; with uncoupler present, however, this acidification is abolished and, instead, there is an alkalinisation corresponding to the predicted consumption of 1 H^+ per electron donated to oxygen.

The reported measurements of H^+ translocation by cytochrome *c* oxidase in reconstituted vesicles have recently been the subject of strong criticism [7,8], both with regard to the experiments in particular and to the validity in general of the reconstituted system for such studies. Whilst most of the specific points have been answered [9], an important remaining discrepancy concerns the very slow decay of the cytochrome-*c*-induced pulse of H^+ ejected from oxidase vesicles (see, e.g., Refs. 3, 4 and 6) which is considered by Mitchell and Moyle [7] and by Papa et al. [8] to argue against genuine H^+ translocation and to indicate instead

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Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; TPP^+, tetraphenylphosphonium$^+$.

that the ejected protons are released from groups at the external surface of the membrane. We present below a quantitative analysis of the decay kientics of the ejected H^+ pulse and endeavour to resolve the discrepancies concerning the decay rate.

Considering the reconstituted oxidase system in general, it must be conceded that, whilst commonly used as a simple model system and although relatively well-characterised, oxidase vesicles do have some intrinsic complexities (see Ref. 10 for a review) which must be taken into account when interpreting experiments of the type mentioned above. A particularly important feature of this system regarding experiments on H^+ pumping is the structural heterogeneity of the vesicles within a preparation. In the work described below, we examine the characteristics of H^+ translocation by cytochrome *c* oxidase with regard to a detailed analysis of the oxidase vesicle system.

Materials and Methods

Bovine heart cytochrome *c* oxidase was reconstituted into phospholipid vesicles as described previously [4]. For vesicles lacking internally trapped Hepes, the procedure was the same except that the sonication step was performed with 40 mg/ml asolectin in 0.1 M KCl/24.5 mM potassium cholate/2 mM Hepes (pH 7.4) and the dialysis was carried out against 0.1 M KCl. H^+ translocation was measured as the change in absorbance of Phenol red at 556.5–504.5 nm using an Aminco DW-2a spectrophotometer fitted with a magnetic stirring device. H^+ extrusion decays were fitted to theoretical curves using a programme based on that of Duggleby [11] which was adapted for a CMB 2001 computer fitted with a BBC Servogor plotter. Measurement of tetraphenylphosphonium distribution was carried out using a TPP^+-sensitive liquid membrane electrode constructed locally according to Ref. 12. Care was taken to control for the amount of TPP^+ binding to the vesicle membranes (see Ref. 5).

Gel filtration of cytochrome *c* oxidase vesicles was performed at 4°C using a Sepharose 4B column (35 × 2 cm); 75 mM choline chloride/25 mM KCl/10 mM Hepes (pH 7.4) was used for both equilibration and elution. The column was calibrated using markers of known molecular weight.

Ficoll density gradient centrifugation analysis was carried out at $110000 \times g_{av}$ and 4°C for 20 h in an 8 × 16 ml swing-out rotor of an MSE Superspeed 65 centrifuge.

Protein was measured according to the method of Lowry et al. [13]. Phospholipid was determined from its total phosphorus content according to the method of Chen et al. [14].

Ferrocytochrome *c* and asolectin were prepared as described in Ref. 4. TPP^+ chloride was from Aldrich Chemical, CCCP and valinomycin from Sigma Chemical and Ficoll from Pharmacia Fine Chemicals; other substances were of the highest purity commercially available.

Results and Discussion

The kinetics of back-flux of protons extruded from cytochrome c oxidase vesicles

The extravesicular acidification which occurs when ferrocytochrome *c* is oxidised by reconstituted cytochrome *c* oxidase vesicles and which has been interpreted as active H^+ extrusion decays slowly to give a net alkalinisation corresponding to 1 H^+ consumed per electron passing to oxygen (see Fig. 1A). Provided that valinomycin-catalysed K^+ movement were fully competent in dissipating any transmembrane electrical potential, then the decay of the proton pulse, as the protons equilibrate with the internal space, should be limited only by the membrane H^+ permeability. When a similarly sized acidification of the external medium is induced by adding a pulse of acid, this also decays as the protons equilibrate with the intravesicular buffer and, by the above reasoning, it should do so with a half-time similar to that of the decay of the extruded proton pulse. In fact, the pulse of extruded H^+ decayed much more slowly (half-time, 140 s; see Figs. 1A and 3) than a pulse of added acid (half-time approx. 20 s, see Fig. 3) under these conditions. This slow decay, which is characteristic of most of the reports of H^+ pumping by cytochrome *c* oxidase vesicles as measured with ferrocytochrome *c* pulses (see, e.g., Refs. 3, 4 and 6), has been held [7,8] to contradict genuine H^+ translocation and to indicate instead that the cytochrome-*c*-induced acidification results from the

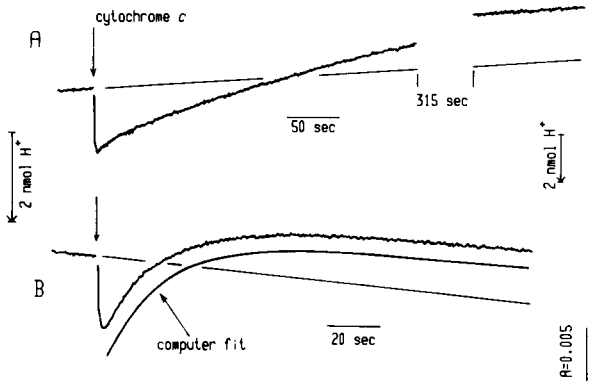

Fig. 1. Cytochrome-*c*-induced extrusion of H^+ from cytochrome *c* oxidase vesicles in the presence of valinomycin. The changes in pH of a suspension of cytochrome *c* oxidase vesicles (0.6 nmol haem *a*) in 75 mM choline chloride/25 mM KCl/50 μM Phenol red (pH 7.4) (total volume, 1.4 ml) in the presence of 0.09 (trace A) or 0.80 (trace B) nmol valinomycin per μmol phospholipid were measured spectrophotometrically at 24°C as the change in Phenol red absorbance. At the points indicated, 2.3 nmol ferrocytochrome *c* were added. The curve below trace B is a computer-generated fit to the decay of the pulse of extruded H^+ (see text). The square-ended lines to the left and right of the traces show the changes in absorbance induced by 2 μl 0.5 mM oxalic acid in the absence and presence, respectively, of 3.5 μM CCCP.

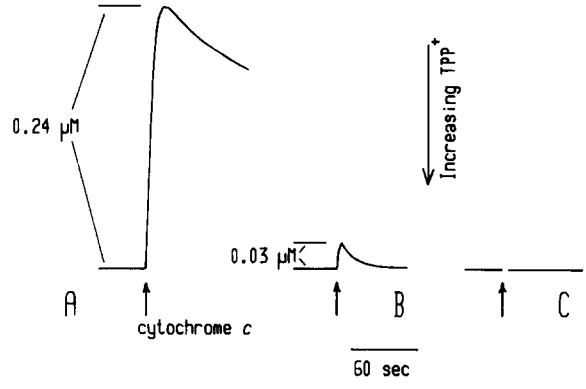

Fig. 2. Electrical-potential-linked uptake of TPP^+ by suspensions of cytochrome *c* oxidase vesicles. Cytochrome *c* oxidase vesicles (0.6 nmol haem *a*) were suspended in 75 mM choline chloride/25 mM KCl/10 mM Hepes (pH 7.4) (total volume, 1 ml) at 24°C with no further additions (A) or in the presence of valinomycin at concentrations of 0.09 (B) or 0.8 (C) nmol/μmol phospholipid. TPP chloride was present at a concentration of the free species of 0.65 μM. 2.5 nmol cytochrome *c* was added at the points indicated by the arrows, and the resulting change in the concentration of TPP^+ was measured electrometrically as described in the Materials and Methods section. These concentration changes, calculated from the electrode potential changes using the Nernst equation, are shown beside the traces.

dissociation of protons from the vesicle surface. In these measurements, valinomycin was used at concentrations between 0.04 and 0.2 nmol per μmol phospholipid to catalyse electrically dissipative K^+ movement and thus to prevent a build-up of transmembrane electrical potential. Whilst our own measurements (not shown) and those of others (see, e.g., Ref. 15) have shown that valinomycin at these concentrations is fully competent in allowing the passive flux of K^+ down its concentration gradient, there are indications that much larger concentrations of this ionophore are needed to allow full dissipation of the electrical potential resulting from proton pumping in reconstituted vesicles [16]. To investigate the efficacy of valinomycin in counterion transport in H^+-pumping oxidase vesicles the electrical gradient resulting from cytochrome *c* oxidation was measured from the accumulation of the permeant cation TPP^+ [17] (see Fig. 2). In the absence of valinomycin, a clear TPP^+ uptake occurred indicating an internally negative potential of 115 mV, assuming an internal volume of 1.8 μl/mg lipid (calculated from the data of Fig. 5, see below). In the presence of valinomycin at the concentration used for the experiment of Fig. 1A, there was only an incomplete dissipation of the electrical potential following cytochrome *c* oxidation, as reflected by a small residual uptake of TPP^+. When the valinomycin concentration was increased approx. 9-fold, however, the TPP^+ uptake was fully abolished.

To test the possibility that an insufficiency of valinomycin might also be the cause of the anomalous decay kinetics of the extruded proton pulse, the half-time of this decay in the presence of valinomycin at various concentrations was measured (see Fig. 3). Indeed, this decay rate increased with increasing valinomycin concentrations until, with 0.8 nmol valinomycin per μmol lipid (i.e., that concentration where TPP^+ uptake was fully abolished, see Fig. 2), the pulse of extruded H^+ decayed with a half-time of 18 s (see also Fig. 1B) very close to that for a pulse of added acid (15 s).

The accelerated decay cannot be ascribed to valinomycin causing an increased H^+ conductance of the vesicle membranes; although there was a small increase in the rate of decay of the acid

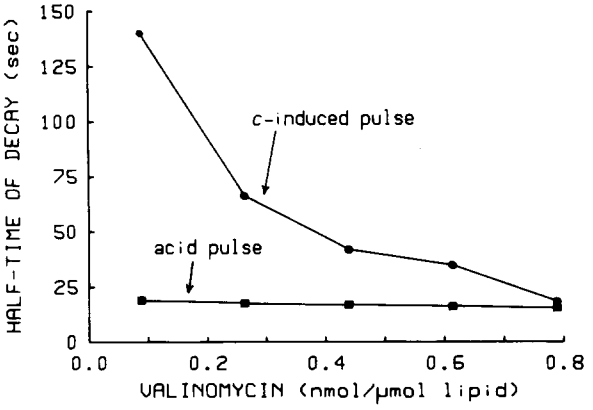

Fig. 3. The kinetics of decay of proton pulses in suspensions of cytochrome *c* oxidase vesicles. Cytochrome *c* oxidase vesicles were suspended as described for Fig. 1. Either 2.5 nmol ferrocytochrome *c* or 2 nmol oxalic acid were added in the presence of valinomycin at the concentration shown. The resulting acidifications and their decays were monitored spectrophotometrically as the change in Phenol red absorbance. These measurements were then repeated in the presence of 3.5 μM CCCP; the extent of the changes in pH with CCCP present were taken as baselines in order to calculate the half-times of decay of the acidifications occurring in the absence of CCCP. These half-times are plotted as a function of the molar ratio of valinomycin to lipid, for the acidifications resulting from oxidase turnover (●) or from acid addition (■).

pulse, this was maximally only 20%, compared to a maximal increase of 700% for that of the extruded protons.

The above findings may be rationalised as follows. When present at the lower concentrations normally used for these measurements, there is approx. 1 molecule of valinomycin per oxidase molecule, i.e., less than 1 valinomycin molecule per vesicle (see below). When the oxidase turns over, however, eight charges must move almost simultaneously if full electroneutrality is to be preserved, as four internal protons are removed to reduce oxygen and four more are extruded. This leads to the possibility that, under these conditions, a transient membrane potential may develop, as indeed is indicated by the experiment of Fig. 2. This potential may cause an alteration in the membrane such that K^+ transport by valinomycin is hindered, and thus causing the decay of the H^+ extrusion to be slowed relative to that of an acid pulse into vesicles which have not undergone oxidase turnover. Indeed, Shinitzky and co-workers [18] have recently reported increased viscosity of lipid vesicle membranes induced by transmembrane electrical potentials in the range of those measured here and that this increase apparently persists after the potential has been removed. Such an increased viscosity in the vesicle membranes after enzyme turnover and the resulting decreased mobility of valinomycin might account for the observations described above. Clearly, when higher concentrations of valinomycin were used, the full dissipation of the electrical gradient resulting from oxidase turnover would have been facilitated and thus this effect avoided (see Fig. 1B).

It is noteworthy that, under conditions where the membrane potential was fully abolished (i.e., those for Fig. 1B), the extrapolated ratio of H^+ extruded per electron increased to 0.95 (cf. that of 0.70 for Fig. 1A; see also below). This is consistent with a slight underestimation of this ratio in the latter case, owing to the small residual potential (see Fig. 2) causing a rapid back-flux of some of the extruded H^+.

Valinomycin and K^+ may be replaced totally as the charge neutralisation system if the medium is supplemented with TPP^+, at a concentration of 1 mM [5]. As shown in Fig. 4, also under these conditions the pulse of extruded H^+ decays with a half-time (16 s) similar to that of equilibration of an acid pulse under the same conditions (half-time 14 s, not shown). This is consistent with the above rationale, considering that the availability of TPP^+ was greater owing to its higher concentration and to the fact that a large proportion of this substance may partition into the vesicle membranes [5]. We conclude that with appropriate facilities for transmembrane charge neutralisation the characteristics of the pulse of H^+ ejected from cytochrome *c* oxidase vesicles on cytochrome *c* oxidation are entirely consistent with active H^+ pumping from the vesicle interior.

Having demonstrated decay kinetics for the cytochrome *c*-induced H^+ extrusion in accordance with the H^+ permeability of the vesicle membranes, it was of interest to examine these kinetics more quantitatively. The decays of the H^+ pulses shown in Figs. 1B and 4 were subjected to computer analysis using a curve-fitting programme. A decay curve describing a single exponential process, as might be expected for the decay of pH

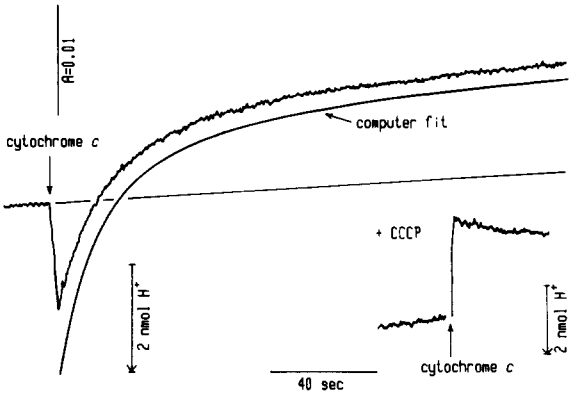

Fig. 4. Cytochrome-*c*-induced extrusion of H^+ from cytochrome *c* oxidase vesicles in the presence of TPP^+. Cytochrome *c* oxidase vesicles (0.7 nmol haem *a*) were suspended in 25 mM KCl/75 mM choline chloride/50 μM Phenol red (pH 7.4) in the presence of 1 mM TPP chloride at 24°C. Changes in the pH of the suspension caused by the oxidation of 2.9 nmol of cytochrome *c* were recorded as for Fig. 1. The line below the trace shows a computer-generated fit to the decay of the pulse of extruded H^+. The square-ended lines show the changes in absorbance induced by 2 μl 0.5 mM oxalic acid in the absence and presence of 3.5 μM CCCP as indicated.

gradients across vesicle membranes all having the same, ohmic H^+-conductance properties (see Refs. 19 and 20), gave a poor fit (not shown). With both valinomycin (0.8 nmol per μmol) and TPP^+ as counterion, however, a good fit was obtained to a curve given by an expression of the type:

$$y = y_0' e^{-k_1 t} + y_0'' e^{-k_2 t}$$

where y is the extent of the acidification at time t, i.e., at $t = 0$, $y = (y_0' + y_0'')$, and k_1 and k_2 are the first-order rate constants for the two components of the decay. (N.B. The half-time values given in Fig. 3 are for the entire decay of the proton pulse and not for either of these individual components.)

These theoretical curves are shown below the decays in Figs. 1B and 4. In both cases an initial rapid decay was followed by a slower phase. With valinomycin, the best fit was obtained using $k_1 = 0.07\ s^{-1}$ and $k_2 = 0.026\ s^{-1}$; with TPP^+, these values were 0.05 and 0.023 s^{-1}, respectively. Extrapolation of the theoretical curves indicated that the full acidification resulting from H^+ extrusion (i.e., the value of y at $t = 0$) corresponded to 0.95 and 0.96 H^+ per electron with valinomycin and TPP^+, respectively (cf. the measured values of 0.72 and 0.67 H^+ per electron).

The biphasicity of the decay kinetics cannot be due to a residual transmembrane electrical potential; under conditions similar to those of Fig. 1B no cytochrome-*c*-induced uptake of TPP^+ occurred (see Fig. 2). A complication in the decay kinetics caused by a slow phase of cytochrome *c* oxidation was also excluded as complete oxidation occurred within 2 s under the conditions of Figs. 1B and 4 (not shown). A further explanation for the biphasic decay kinetics might be back-flux of extruded protons into subpopulations of vesicles having, for instance different H^+-conductance characteristics or transmembrane proton gradients following oxidase turnover; such effects might result, for example, from a heterogeneous distribution of enzyme molecules amongst the vesicles. Clearly, a complete understanding of this and other kinetic aspects of the cytochrome *c* oxidase H^+ pump requires a thorough knowledge of the various subpopulations within the vesicle preparation and the distribution of oxidase molecules amongst these. The following characterisation of the oxidase vesicle preparations was thus carried out.

Characterisation of cytochrome c oxidase vesicle preparations with regard to vesicle size and composition

Cytochrome *c* oxidase vesicles prepared by the technique of cholate dialysis are highly heterogeneous with respect to vesicle size (see, e.g., Refs. 21 and 22). In Fig. 5 are shown the variation of a number of structural parameters of oxidase vesicles with their size, in subpopulations eluted from a Sepharose 4B column (see the Materials and Methods section). This shows that the larger vesicles (diameter ranging from 30 to 62 nm) represent the bulk of the total lipid (79 mol%) and protein (83 mol%). The very small vesicles (diameter less than 30 nm) on the other hand, whilst being numerically prevalent, account for minor proportions of these constituents (21 mol% of the lipid and 17 of the protein).

A particularly important structural parameter is the number of oxidase molecules (an oxidase molecule is defined here as the subunit complex which contains two haems and two copper atoms)

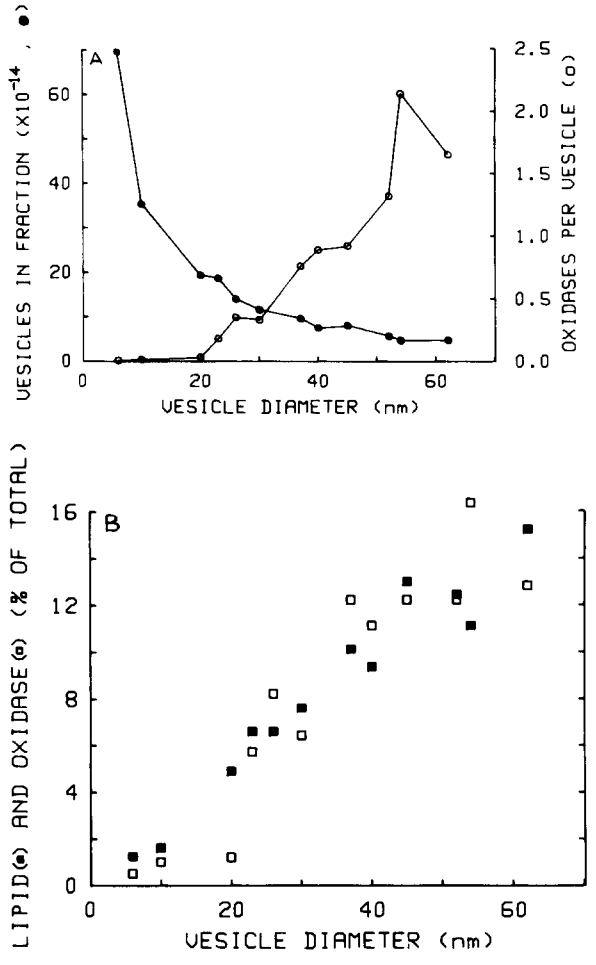

Fig. 5. Structural characteristics of subpopulations of cytochrome *c* oxidase vesicles. Cytochrome *c* oxidase vesicles (3 ml containing 10.5 nmol oxidase) were resolved into 1.5 ml fractions using a Sepharose 4B column and the amounts of protein (□) and lipid (■) in the fractions are shown (B) as the percent proportion (%) of their total amounts in the vesicle preparation. The number of vesicles in a particular fraction (●) was calculated by dividing the total number of lipid molecules in that fraction by the number in a single vesicle, assuming the membrane thickness to be 3.7 nm and the area occupied by a single phospholipid head group to be 0.74 and 0.61 nm^2 in the outer and inner leaflets of the bilayer, respectively [30]. This value was divided by the total number of oxidase molecules in that fraction to give the ratio of oxidase molecules per vesicle (○) (A).

contained in a single vesicle. In the case of the more abundant vesicle fractions (i.e., those having a diameter greater than 30 nm) the ratio of oxidase molecules to vesicles varies between 0.8 and 2. For the smaller vesicles, this ratio is much lower indicating that incorporation of the enzyme into these vesicles becomes physically hindered, either by the small internal space or by the high degree of curvature of the vesicle membranes. The value for the number of oxidase molecules per vesicle given above represents an average for all the vesicles in a particular subpopulation. It cannot be excluded, however, that in each fraction the oxidase molecules were concentrated within a small number of equally sized vesicles, causing a large error in the calculated oxidase to vesicle ratio. To investigate this possibility, oxidase vesicle preparations were analysed by centrifugation through a Ficoll density gradient; clearly, if a considerable proportion of the vesicles were indeed not associated with oxidase, this should be clear from comparison of the sedimentation profiles for lipid and protein. The profiles from a typical experiment are shown in Fig. 6. In all fractions which contained oxidase, the enzymic activity was stimulated 4- to 6-fold by a protonophore and valinomycin, indicating that all the oxidase was still contained in intact vesicles after centrifugation. The analysis of Fig. 6 indicates that approx. 86% of the lipid was associated with cytochrome *c* oxidase and that only 14%

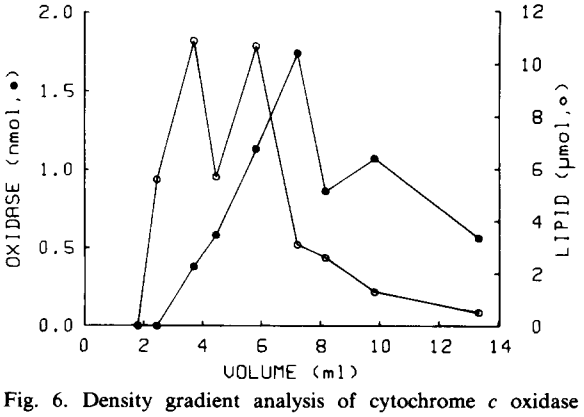

Fig. 6. Density gradient analysis of cytochrome *c* oxidase vesicles. Cytochrome *c* oxidase vesicles (0.85 ml containing 3.2 nM oxidase) were layered onto a discontinuous Ficoll gradient composed of 2.5 ml layers of 55 mM KCl/20 mM Hepes (pH 7.4), containing Ficoll at concentrations of 5, 6, 8, 10 and 15%. The sample was then centrifuged, fractionated by peristaltic suction and assayed for lipid and protein as described in the Materials and Methods section. The gradient fractions are plotted as their position in the total volume of the gradient. The zero volume point represents the top of the gradient.

represented 'empty' vesicles. This latter value is very close to that which can be calculated from the data of Fig. 5 (18%) assuming a fully homogeneous distribution of oxidase molecules throughout the vesicle preparation. When vesicles prepared under identical conditions but in the absence of oxidase were subjected to such a centrifugation, all the lipid was recovered within the uppermost 1.8 ml of the gradient, i.e., above the point where protein was detectable in the centrifugation shown in Fig. 6. Consequently, any contamination of the oxidase-containing fractions in Fig. 6 with 'empty' vesicles is extremely improbable. The above results would thus indicate an even distribution of enzyme molecules throughout the vesicle preparation and would entail that, from the data of Fig. 5, approx. 70% of the oxidase molecules are contained individually in separate vesicles and that the other 30% are in vesicles containing two enzyme molecules.

These findings are at variance with those of Carroll and Racker [23] who reported that all the cytochrome *c* oxidase in their vesicle preparations was concentrated in 10% of the vesicles. Their reconstitution procedure, however, was to add cytochrome *c* oxidase to preformed phospholipid vesicles in the presence of a low concentration of detergent. The clear difference between the preparative techniques used there and here may account for this discrepancy.

The observation that approx. 70% of the reconstituted vesicles in our preparations contain one oxidase molecule has the noteworthy consequence that, following reconstitution, the 'monomeric', two haem-two copper complex appears to be competent in H^+ pumping. An alternative interpretation would be that the 70% of oxidase molecules contained singly in vesicles have a very low activity in electron transfer owing to their being monomeric (see, e.g., Refs. 24 and 25) and that the remainder, which may be dimeric, have a much higher electron transport activity [24,25] and thus oxidise the bulk of the cytochrome *c*. This is made very unlikely by our observation (not shown) that the maximal turnover number of the oxidase in reconstituted vesicles is close to that in intact mitochondria under the same conditions, indicating that practically all the reconstituted oxidase molecules are fully active. A further possibility would be that the monomeric reconstituted oxidase can oxidise cytochrome *c* but might be inactive in H^+ translocation. This would entail that the remaining 30% of the oxidase molecules must each translocate on average approx. 3 H^+ per electron, i.e., have a higher stoichiometry than any of those reported for oxidase in intact mitochondria (see, e.g., Refs. 1, 26 and 27).

A further noteable structural characteristic of oxidase vesicles regarding experiments on energy coupling concerns the membrane orientation of the enzyme in the various subpopulations. We have shown elsewhere [22] that, with vesicles prepared by cholate dialysis, approx. 85% of the reconstituted oxidase molecules have the mitochondrial orientation and that this value is essentially invariant throughout the vesicle preparation.

It has been suggested [4,28] that certain characteristics of H^+ extrusion from cytochrome *c* oxidase vesicles might be explained by a large internal alkalinisation occurring in the oxidase vesicles following cytochrome *c* oxidation; such an alkalinisation might also account for the complexity of the decay kinetics mentioned above by inducing an increased permeability to H^+ in some of the vesicles (see Ref. 29). The fact that the oxidase molecules appear to be homogeneously distributed throughout the vesicle suspension (see above) allows us to calculate the cytochrome-*c*-induced intravesicular pH change, resulting from a low number of enzyme turnovers for the various subpopulations of oxidase vesicles (see Fig. 7). The pH changes were calculated using the determined values of 19.3 mmol H^+/pH unit per mol for the buffering power of the phospholipid and 47.6 nmol H^+/pH unit per μl of internal space, as the buffering power of the internally trapped Hepes (see the Materials and Methods section). The internal volume of each sub population of vesicles was calculated from the data of Fig. 5 assuming a bilayer thickness of 3.7 nm [30]. These values together with those calculated for the molar quantities of internally facing phospholipid in each fraction thus allowed the pH change caused by the oxidation of the quantity of cytochrome *c*, indicated in Fig. 7 to be calculated. It is clear that in no case does a drastic alkalinisation occur, the largest change (i.e., that with 3 turnovers in the

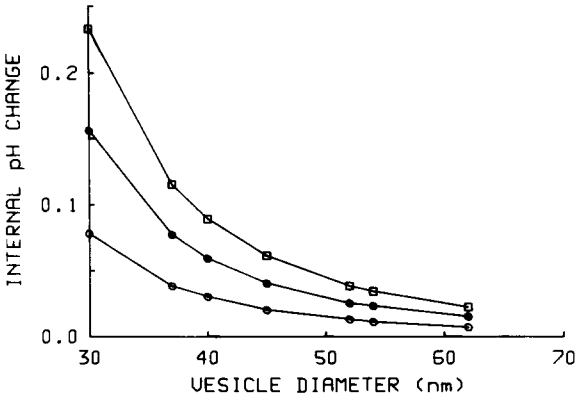

Fig. 7. Internal alkalinisation induced by enzyme turnover in sub-populations of cytochrome *c* oxidase vesicles. The increase in the internal pH of vesicles, having the diameter shown, as a result of one (○), two (●) or three (□) turnovers of the cytochrome *c* oxidase molecules (i.e., from the oxidation of four, eight or twelve molecules of cytochrome *c* per enzyme molecule) was calculated as described in the text. Vesicles with a diameter of 54 nm were assumed each to contain two oxidase molecules; all others were assumed to contain one.

smallest vesicles analysed) being only 0.23 pH units.

In the above treatment, a complication of the kinetics of back-flux of extruded H^+ by a residual transmembrane potential, a slow phase of cytochrome *c* oxidation or a large intravesicular alkalinisation have been considered and excluded. This indicates that double-exponential kinetics (see Figs. 1B and 4) are a genuine feature of the extruded H^+ pulse decay. In this regard, it is noteworthy that a similar type of biphasic kinetics has been observed for the decay of a transmembrane proton gradient formed by proton pumping via bacteriorhodopsin in subbacterial vesicles from *Halobacterium halobium* [31]. In addition, Mitchell [32] has derived a theoretical model predicting that the decay of a proton gradient across a coupling membrane should behave as the sum of two exponential processes, as found here. It may be, therefore, that such double-exponential kinetics are a general feature of the decay of actively imposed proton gradients, at least in small vesicles.

Acknowledgements

The work reported here was supported by the grant 3739-080 from Schweizerischem Nationalfonds and by the Sandoz Stiftung. P.S. O'S. gratefully acknowledges the award of a Royal Society postdoctoral fellowship. We are grateful to the Roche Foundation for the award of a fellowship to J.B.C. We thank Dr. M. Solioz for providing TPP^+-sensitive membranes.

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