Kinetic studies on cytochrome *c* oxidase inserted into liposomal vesicles

Effect of ionophores

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(Received 29 April 1982/Accepted 17 August 1982)

Cytochrome *c* oxidase from ox heart was inserted into artificial liposomal vesicles obtained by sonication of purified soya-bean phospholipids. The cytochrome oxidase vesicles showed a respiratory control ratio of about 2. Spectroscopic properties in the visible and Soret regions and kinetics of CO binding are similar to those of the soluble oxidase. The catalytic efficiency of the cytochrome oxidase vesicles in oxidizing cytochrome *c* increases as a result of the formation of the 'pulsed' form of the oxidase and of the presence in the reaction mixture of carbonyl cyanide *p*-trifluoromethoxy-phenylhydrazone and nonactin. Analysis of the experimental results obtained under several conditions supports the conclusions that: (i) the alkalization of the internal microenvironment in the liposomal vesicle is not by itself responsible for the decrease in catalytic activity; (ii) the electrical potential difference created during turnover by proton consumption and/or pumping through the liposome wall is an important mechanism of control in the chain of events leading to the oxidation of external cytochrome *c*.

It is nowadays accepted that cytochrome *c* oxidase (ferrocytochrome *c*-oxygen oxidoreductase, EC 1.9.3.1) exists in two functionally distinct states, called 'resting' and 'pulsed', which also differ in some spectroscopic properties (Antonini *et al.*, 1977; Brunori *et al.*, 1979a, 1981; Colosimo *et al.*, 1981). Resting oxidase (R) corresponds to the enzyme as obtained by the isolation procedure(s), whereas the pulsed (P) state is generated from the resting one either during turnover or by reaction of reduced oxidase with O₂. Some characteristic differences between these two states involve: (i) the internal electron transfer step leading to reduction of cytochrome *a*₃, which is faster in pulsed oxidase; (ii) the redox potential of cytochrome *a*, which is more positive (by approx. 20 mV) in pulsed oxidase; (iii) the absorption spectrum in the α -band and Soret regions, which in the pulsed oxidized state is similar to that of the so-called 'oxygenated' derivative; (iv) the e.p.r. spectrum, which shows a new signal at $g = 5$ (Beinert *et al.*, 1978).

A possible interpretation of the significance of these two states as a regulatory mechanism, pre-

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viously proposed (Antonini *et al.*, 1977), was substantiated by the results of experiments performed on submitochondrial particles (Bonaventura *et al.*, 1978). A much clearer picture of the physiological role (if any) of both the resting and the pulsed cytochrome *c* oxidase may be obtained, however, if the system under investigation contains the essential structure of the energy-conversion phenomena occurring in mitochondria, i.e. a closed proteolipid membrane.

In the present paper we report a series of experiments on cytochrome *c* oxidase inserted into liposomal vesicles, undertaken with the double aim of: (i) extending our previous experiments on soluble oxidase to a model system that, although oversimplified, is much closer to the situation *in vivo*; (ii) finding a possible relationship between the flux of electrons and/or ions across the liposome wall and the different functional states of cytochrome *c* oxidase. This may in principle shed light on the structural basis of the interconversion between the pulsed and the resting states, as well as on the regulatory mechanisms tuning the proton and electron fluxes in mitochondria.

Experimental

Materials

Asolectin was obtained from Associated Concentrates (Woodside, NY, U.S.A.). Cytochrome *c* (type VI) and nonactin were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone was a gift from Professor T. Galeotti (Catholic University of Rome, Rome, Italy). All other reagents were of analytical grade.

Cytochrome *c* oxidase was purified from ox heart by the procedure of Yonetani (1961).

Spectrophotometry

Spectrophotometric and stopped-flow measurements were made respectively with a thermostatically controlled Cary 219 instrument and with a Durrum-Gibson apparatus equipped with a 2 cm observation chamber and having a dead-time of 2–4 ms. Flash-photolysis experiments were performed with the instrument described previously (see Brunori & Giacometti, 1981).

Electron microscopy

Microscopical observations were made with a Siemens 1A electron microscope.

Determination of cytochrome *c* and cytochrome *c* oxidase

The cytochrome *c* and cytochrome *c* oxidase concentrations were determined spectrophotometrically by using the molar absorption coefficients of the reduced forms, respectively $27.6 \text{ mm}^{-1} \cdot \text{cm}^{-1}$ at 550 nm and 21 (total haem) $\text{mm}^{-1} \cdot \text{cm}^{-1}$ at 605 nm (Yonetani, 1961; Brunori *et al.*, 1979).

Preparation of cytochrome oxidase vesicles

Soya-bean phospholipids were partially purified from Asolectin as described by Hinkle *et al.* (1972) and sonicated by the procedure of Wrigglesworth (1978). To minimize the oxidation of phospholipids and the formation of larger aggregates, which are time-dependent phenomena, preparations were always used within 2–3 days after sonication.

The cytochrome oxidase vesicles were obtained by following several methods described in the literature (Hinkle *et al.*, 1972; Wrigglesworth, 1978). Each preparation was submitted to the tests for respiratory control ratio, catalytic activity and orientation of the enzyme with respect to the bulk solution. To avoid over-heating and oxidation during sonication, the preparation was kept cold and under a stream of N_2 .

The respiratory control in suspensions of cytochrome oxidase vesicles, defined as the ratio between the rates of O_2 consumption after and before

addition of ionophores, was measured by the classical polarographic method (Hinkle *et al.*, 1972) with a Clark-type oxygen electrode (Rank Brothers, Cambridge, U.K.). Alternative assays, based on the steady-state concentration of cytochrome c^{3+} determined spectrophotometrically (Wrigglesworth, 1978), or on the rate of aerobic oxidation of reduced cytochrome *c* in rapid-mixing experiments before and after addition of the ionophores, gave essentially the same results.

The catalytic activity of cytochrome oxidase vesicles was calculated from the initial velocity of cytochrome *c* oxidation by the method of Yonetani (1967).

The orientation of the enzyme in the vesicles (i.e. the fraction of molecules of oxidase having the cytochrome *c*-reacting site exposed toward the bulk solution) was assessed by differential spectroscopy by the procedure of Wrigglesworth (1978). This is based on the use of *NNN'*-tetramethyl-*p*-phenylenediamine as a permeable redox mediator, capable of reducing in a short time all the oxidase molecules regardless of their orientation. Thus, whereas ascorbate and cytochrome *c* under anaerobic conditions bring about only partial reduction of the oxidase in the vesicles, addition of tetramethyl-*p*-phenylenediamine to the external medium leads to complete reduction of the oxidase, as shown by the increase of absorbance at 605 nm. Addition of Triton X-100, which disrupts the vesicles, also results in a complete reduction of the oxidase and thus makes any subsequent addition of tetramethyl-*p*-phenylenediamine ineffective.

In order to prepare cytochrome oxidase vesicles with an internal pH indicator, sonication was performed in the presence of 5 mM-Phenol Red. The external dye in the suspension was eliminated by passage through a Sephadex G-25 column, just before use.

Results

Characterization of cytochrome oxidase vesicles and orientation of the oxidase

Plate 1 shows an electron micrograph of a typical preparation of cytochrome oxidase vesicles. The homogeneity of the population is fairly good, 80% of the vesicles ranging between 20 and 40 nm in diameter. From the observed diameters of the vesicles and the average head area of a phospholipid molecule (approx. 0.6 nm^2), one may estimate that the liposomal vesicles contain between 500 and 5000 molecules in their internal layer. Taking the molecular weight of dimeric cytochrome *c* oxidase as 240 000 and that of a medium-sized phospholipid molecule as 1000, at a lipid/protein weight ratio of 20:1, an average of one oxidase dimer per vesicle may be calculated.

40 nm

EXPLANATION OF PLATE 1

Electron micrograph of cytochrome oxidase vesicles

The preparation had been sonicated in an MSE sonicator for 1 min/ml at a peak-to-peak energy of $6.5\mu\text{m}$. Negative staining was performed with 1% ammonium molybdate. Formvar/carbon grids (300 mesh) were floated on the surface of a drop of sample and then stained.

The fraction of the oxidase molecules that expose cytochrome *a* to the external medium, and therefore to externally added cytochrome *c*, was found to be about 75%, as measured by differential spectroscopy in the α -band region (see Table 1). The same proportion was found from measurements made in the Soret region provided that the difference spectra were corrected for the cytochrome *c* contributing in this region. Essentially identical results were obtained for different preparations, obtained by different incorporation procedures.

The reaction of cytochrome oxidase vesicles with CO was investigated by flash photolysis (Brunori & Giacometti, 1981). The kinetic difference spectrum of CO binding to cytochrome *c*/ascorbate-reduced cytochrome oxidase vesicles is given in Fig. 1, where it is compared with and normalized to the static difference spectrum obtained for the solubilized enzyme. It is clear that incorporation of the enzyme into a lipid bilayer in no way modifies this spectrum. Moreover the second-order combination rate constant for the reaction of the oxidase vesicles with CO (3×10^4 – $3.4 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$) is very similar to that characteristic for the detergent-solubilized enzyme (Greenwood *et al.*, 1974). On addition of tetramethyl-*p*-phenylenediamine an increase of about 20% was observed in the signal amplitude at 430nm, but the rate remained unchanged. This increase may be interpreted in terms of previously oxidized oxidase being reduced and thus participating in the CO-binding process. This experiment yields a value for the cytochrome *a* outside/cytochrome *a* inside orientation ratio of 4:1, in agreement with the values given in Table 1 derived from differential spectroscopy in the α -band region.

Given the impermeability of liposomes to cytochrome *c*, it is clear that the oxidase molecules exposing the cytochrome *a* to the interior of the vesicles will not contribute to any significant extent to the electron-transfer reactions followed by stopped-flow experiments and described in the next sections.

Few-turnover experiments

A number of experiments were performed with the mixing in the stopped-flow apparatus of oxidase incorporated into liposomal vesicles with reduced cytochrome *c*, in an aerobic buffer system containing KCl. The experiments were performed by following the protocol previously devised to follow the oxidation of cytochrome *c* either by resting oxidase (R) or by the more active pulsed form (P) (Antonini *et al.*, 1977; Brunori *et al.*, 1979).

The relative concentrations of oxidase, cytochrome *c* and ascorbate were chosen so that, during the time of the experiment, only a small number of enzyme turnovers was involved in the complete oxidation of cytochrome *c*.

Fig. 1. Reaction of CO with cytochrome oxidase vesicles. The kinetic difference spectrum was obtained by flash-photolysis experiments performed on a suspension of cytochrome oxidase vesicles. The reaction was between cytochrome oxidase vesicles (total haem $1 \mu\text{M}$) and 1 mM -CO in the presence of $2 \mu\text{M}$ -cytochrome *c* and 8 mM -ascorbate in 50 mM -potassium phosphate buffer, pH 7.4, and the temperature was 20°C . The continuous line shows the kinetic difference spectrum for the soluble oxidase (taken from Greenwood *et al.*, 1974).

Table 1. Orientation of cytochrome *c* oxidase inserted into liposomal vesicles

Lipid/protein ratio	Incorporation method	Orientation (% of externally exposed cytochrome <i>a</i> measured from the α -band region)
(13–8):1	Sonication*	74 ± 2
20:1	Sonication*	79 ± 1
20:1	Dialysis†	73 ± 10
20:1	Sonication‡	82 ± 1
30:1	Dialysis†	70 ± 3

* Method of Wigglesworth (1978).

† Method of Hinkle *et al.* (1972).

‡ Method of Nicholls (1978).

The results of several experiments may be summarized as follows. (a) No burst phase was resolved, possibly because at the concentrations employed the direct electron transfer from cytochrome *c* to cytochrome *a* is too rapid (and also because of a mixing artifact that obscured the first 10 ms). (b) Subsequent cytochrome *c* oxidation follows an exponential time course (Fig. 2) similar in shape to that observed with either solubilized oxidase or with Keilin–Hartree particles. Thus it is convenient to express it in terms of a pseudo-first-order rate constant. (c) Under identical conditions the pseudo-first-order rate constant is considerably faster for oxidase vesicles as compared with the oxidase in solution. This rate enhancement, which amounts to approx. 5-fold, is attributed to the effect of phospholipids on the activity, as described by others (Vik & Capaldi, 1977). (d) In agreement with previous observations on both the soluble and the membrane-bound oxidase (i.e. in the Keilin–Hartree particles) (Bonaventura *et al.*, 1978), cytochrome oxidase vesicles can be pulsed and the activation corresponds to an increase in rate constant of 3–4-fold under the condition of the experiment (compare Figs. 2a and 2b).

The effect of ionophores on the polarographic

assay of the oxidase in mitochondria and/or liposomal vesicles is well documented, and forms the basis for the determination of the respiratory control index (Eytan *et al.*, 1976; Carroll & Racker, 1977). Therefore the effects of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone and nonactin (Wrigglesworth, 1978; Lauser, 1978) on the rate of aerobic oxidation of cytochrome *c* were examined by the stopped-flow technique. Both ionophores enhance the first-order rate constant for cytochrome *c* oxidation, and this effect is clearly shown in Fig. 3. Nonactin is considerably more effective in that its action is expressed at lower concentrations (approx. 1 μM) and gives a greater rate enhancement. The order of addition yields more information, because addition of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone to cytochrome oxidase vesicles saturated with nonactin has no effect, whereas addition in the reverse order yields an increase in rate that corresponds to the difference between the individual effects of the two ionophores. This may be related to the longer times required for the complete development of the effect of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (Wrigglesworth, 1978).

The rate enhancements reported for carbonyl

Fig. 2. Time course of oxidation of cytochrome *c* by resting (a) and pulsed (b) cytochrome oxidase vesicles in the absence and in the presence of ionophores

The reaction was followed by mixing in the stopped-flow apparatus cytochrome oxidase vesicles (total haem 5 μM) with 25 μM -cytochrome *c* and 1.5 mM-ascorbate, in 50 mM-potassium phosphate buffer, pH 7.4, containing 50 mM-KCl, and the temperature was 20°C. O and ●, Ionophores absent; □ and ■, nonactin (10 μM) added to the cytochrome *c*-containing syringe; △ and ▲, nonactin (10 μM) and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (10 μM) added to the cytochrome *c*-containing syringe. Identical results were obtained if the ionophores were incubated with the cytochrome oxidase vesicles.

Fig. 3. Dependence on ionophore concentration of the rate constant for the aerobic oxidation of cytochrome *c* by cytochrome oxidase vesicles

Experiments were performed by mixing cytochrome oxidase vesicles (total haem 5 μM) in air with 25 μM-cytochrome *c* reduced by 2 mM-ascorbate. The first-order rate constant for cytochrome *c* oxidation was measured as a function of the concentration of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) (○) and of nonactin (●) added to cytochrome oxidase vesicles. Addition of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (10 μM) to a nonactin-saturated sample (●) shows no change, whereas addition of nonactin (4.5 μM) to a sample containing 10 μM-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (●) shows an increase in *k*_{obs}. The reaction mixture was in 50 mM-potassium phosphate buffer, pH 7.4, containing 50 mM-KCl, and the temperature was 20 °C.

cyanide *p*-trifluoromethoxyphenylhydrazone and nonactin are of similar magnitude with resting and pulsed oxidase, as shown in Table 2, where the rate constants under different conditions are reported.

Stopped-flow experiments were performed in which the ionophore was incubated either with cytochrome oxidase vesicles or with reduced cytochrome *c* before the mixing, but under otherwise identical conditions. The rate enhancements were identical in the two cases, showing that the effect of the ionophore develops rapidly, since observation on cytochrome *c* oxidation starts approx. 10 ms after the mixing. This finding shows that the ionophore penetrates very rapidly the bilayer and exerts its effect instantaneously within this time scale (Läuger, 1978).

Whatever the mechanism of the rate enhancement may be, we believe that this result shows that the increase in rate on addition of ionophores is

Table 2. Effects of nonactin and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) on the pseudo-first-order rate constants for the aerobic oxidation of reduced cytochrome *c* by resting and pulsed cytochrome oxidase vesicles

All experimental conditions were as described in the legend to Fig. 2.

Addition(s) ...	Pseudo-first-order rate constant (s ⁻¹)		
	None	Nonactin	Nonactin + FCCP
Resting cytochrome oxidase vesicles	0.7	1.4	1.4
Pulsed cytochrome oxidase vesicles	2.0	5.0	5.0

much faster than the rate at which proton re-equilibration takes place (even in the presence of ionophores). Thus the rate enhancement does not appear to be related to the rate at which protons are made available inside the liposomal vesicle (see the Discussion section).

Many-turnover experiments

A number of experiments were performed in the presence of an excess of ascorbate over O₂, and were followed in time until, after a clearly observable steady state, the system became completely reduced. In order to monitor the production and collapse of a proton gradient across the liposomal membrane, Phenol Red (pK 7.7) was incorporated inside the vesicles, and a buffer at low concentration {5 mM-phosphate and/or -Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid]} was employed.

Experiments performed in the absence of ionophores show (Fig. 4a) that, after a rapid oxidation phase, cytochrome *c* monitored at 550 nm is maintained at a constant level of oxidation (approx. 80%) until O₂ is exhausted, after approx. 210 s. Monitoring the Phenol Red absorption maximum (at 560 nm), we observed no pH changes over the time range corresponding to the transient phase leading to oxidation of cytochrome *c* (which is completed within 1 s). This is not unexpected in view of the buffer concentration used. On the other hand, during the steady-state phase clear pH changes were observed. The increase in pH of the internal medium, indicated by the direction of the absorbance change of the indicator, continues until O₂ is exhausted and the system becomes reduced. Thus, after the end of the steady state, the internal pH decreases again with a half-time of approx. 2 min, which may be taken to represent the rate of proton leakage through the bilayer.

The addition of nonactin at saturating concentrations largely abolished the absorbance change

Fig. 4. Reaction of cytochrome *c* with cytochrome oxidase vesicles

The cytochrome oxidase vesicles contained encapsulated Phenol Red to monitor internal pH changes associated with turnover (●, observations at 560 nm); the time-dependence of the oxidation state of cytochrome *c* was monitored simultaneously at 550 nm (○). (a) An aerobic suspension of cytochrome oxidase vesicles (4.5 μ M) was mixed with a degassed solution of cytochrome *c* (25 μ M) in the presence of 2 mM-ascorbate. (b) Saturating concentrations of nonactin ($\geq 5 \mu$ M) were also present in the oxidase-vesicle-containing syringe. The reaction mixture was in 5 mM-potassium phosphate buffer, pH 7.4, containing 50 mM-KCl, and the temperature was 20°C. The preparation of cytochrome oxidase vesicles had been sonicated in the presence of approx. 2.5 mM-Phenol Red.

at the Phenol Red band, while simultaneously enhancing the catalytic rate of the enzyme, in agreement with what was reported above. The latter point is shown in Fig. 4(b), where, in the presence of nonactin, the steady-state level lies towards oxidized cytochrome *c* and lasts for a shorter time (approx. 185 s), i.e. the enzyme exhausts the available O_2 more rapidly. With carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone under the same experimental conditions as used for Fig. 4(b), a small proton gradient was apparently established during the steady state.

With the purpose of extending these observations into a faster time range, we performed experiments in which tetramethyl-*p*-phenylenediamine and ascorbate were set to concentrations ensuring rapid O_2 consumption. The time course was again followed at two wavelengths.

At 557.2 nm, which is strictly isosbestic for cytochrome *c* oxidation–reduction, the alkalization of the internal pH is observed both in the absence and in the presence of nonactin during turnover (Fig. 5). In the presence of the ionophore the initial rate of the pH change is greater than in its absence, in qualitative agreement with the enhanced activity of the enzyme resulting from addition of

nonactin and/or carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (see Table 2). In its absence the build-up of the pH gradient is linear with time, consistent with zero-order kinetics and impermeability of the vesicles towards protons over the first 15 s. It may be remarked that under the conditions used for Fig. 4(b) no pH change was observed in the presence of nonactin since under low-turnover conditions this ionophore is able to re-equilibrate the internal medium with the bulk phase rapidly enough to cancel any build-up of H^+ gradient. At the end of turnover, when O_2 is exhausted, the proton gradient begins to collapse, and this is clearly much faster in the presence of nonactin (Fig. 5b) (with a half-time of approx. 4 s).

At 550 nm both cytochrome *c* and Phenol Red make significant contributions, and the observed progress curves at this wavelength are also shown in Fig. 5. On the basis of an independent determination of the dye contribution at the two wavelengths we have been able to correct the progress curves to obtain the time course of cytochrome *c* oxidation and reduction (as shown in Fig. 5). Both in the presence and in the absence of nonactin we observe that the profile of the steady state is as described previously for identical experi-

Fig. 5. Reaction of cytochrome *c* with cytochrome oxidase vesicles

Cytochrome oxidase vesicles containing Phenol Red were mixed with ascorbate-reduced cytochrome *c* in the absence (a) and in the presence (b) of nonactin, and the reaction was followed at 550 nm (■) and at 557 nm (●). The O symbols give the absorbance changes at 550 nm corrected for the Phenol Red contribution and thus represent the progress curves for the redox changes in cytochrome *c*. The reaction was between cytochrome oxidase vesicles (total haem 2.5 μM) and 9.5 μM-cytochrome *c* in the presence of 5 mM-ascorbate; 0.33 mM-tetramethyl-*p*-phenylenediamine and 5 μM-nonactin (all concentrations are after mixing) in 50 mM-potassium phosphate buffer, pH 7.4, containing 50 mM-KCl, and the temperature was 20°C. The preparation of cytochrome oxidase vesicles had been sonicated in the presence of 5 mM-Phenol Red immediately before the experiment and freed from the external Phenol Red by column treatment. At 1 h after completion of the experiments the vesicles still contained more than 80% of the initially entrapped dye.

performed with the solubilized oxidase (Brunori *et al.*, 1979a).

Discussion

It has been confirmed that incorporation of ox heart cytochrome *c* oxidase into liposomal vesicles results in an enhancement of activity that, under our conditions, is increased approx. 5-fold as compared with the solubilized enzyme, most probably owing to the effect of phospholipids (Vik & Capaldi, 1977). Moreover the earlier observation that cytochrome *c* oxidase inserted into liposomal vesicles displays a respiratory control (Eytan *et al.*, 1976), thus indicating that the vesicles are impermeable to charged components, has also been reproduced.

Analysis of the distribution of the enzyme and its orientation relative to the bulk solution demands some comments. The number of oxidase dimers (i.e. 2 functional units) per vesicle is not uniquely determined, although on the average a 1-to-1 ratio seems to be predominant. In addition, also, the orientation of cytochrome oxidase is not uniquely determined, and under our conditions approx. 75% of the molecules face the bulk solution with cytochrome *a*, the electron gate into oxidase. This population ratio is not significantly altered by using different incorporation procedures, as shown by the results given in Table 1.

The ligand-binding properties of the oxidase are not affected by incorporation into liposomal vesicles, and the spectral and kinetic properties for the reaction of cytochrome oxidase vesicles with CO are identical with those characteristic of the soluble enzyme. In addition, the amplitude of the CO recombination kinetics, once accurately measured by flash photolysis, may be successfully used to estimate the orientation of the enzyme relative to the bulk solution, in full agreement with the static determination performed by differential spectroscopy.

The topological inhomogeneity of the oxidase population should not represent an obstacle in the interpretation of the stopped-flow experiments. In fact, over the time interval studied, the substantial impermeability of the cytochrome oxidase vesicles to charged macromolecules, such as cytochrome *c*, ensures that the oxidase molecules having cytochrome *a* exposed to the interior of the vesicle will not be reduced and thus will not take part in the electron-transfer processes.

In agreement with preliminary data previously reported (Brunori *et al.*, 1979b), we have shown that cytochrome oxidase vesicles can be pulsed by exposing the reduced form of the enzyme to O₂. The rate constant for the aerobic oxidation of cytochrome *c* by the pulsed cytochrome oxidase vesicles is approx. 3–4-fold greater than that of the resting

ments in the absence of the dye, including a small overshoot when the resting enzyme is used (which is possibly due to spectral changes in this form of the oxidase after the steady state). The maximum steady-state level of oxidation achieved corresponds to 40–45% ferricytochrome *c*, which is in good agreement with estimates from similar experiments

cytochrome oxidase vesicles, similarly to what is found for the enzyme in submitochondrial particles (Bonaventura *et al.*, 1978) and in solution (Antonini *et al.*, 1977).

Most of the results reported in the present paper deal with the effect of two ionophores (namely carbonyl cyanide *p*-trifluoromethoxyphenylhydrazide and nonactin) on the kinetic properties of the pulsed and the resting forms. The effects of these ionophores may be summarized by the results obtained in limited-turnover experiments (Table 2). These show (i) that the maximum rate enhancement for cytochrome *c* oxidation on addition of ionophores is approx. 2.5-fold, which is similar to the respiratory control ratio estimated by the polarographic method (i.e. approx. 2 for our different preparations), and (ii) that this enhancement is the same whether pulsed or resting cytochrome oxidase vesicles are employed.

From the results presented in Fig. 2 we may draw some conclusions on the factors that influence or control the rate at which electrons pass through the enzyme *en route* to the ultimate acceptor, molecular O₂. Attention may be drawn to the linearity of the logarithmic plots depicted in Fig. 2, indicating that under these conditions the oxidation of ferrocytochrome *c* is an exponential process. Thus in limited-turnover experiments (i.e. approximately two turnovers) and in the absence of ionophores we see no evidence for progressive deactivation of either resting or pulsed cytochrome oxidase during cytochrome *c* oxidation. This implies that, starting from the time of our initial observation (approx. 50 ms after mixing), no significant effect of the build-up of any gradient is manifest. What then controls the rate of the cytochrome *c* oxidase reaction and how do ionophores enhance this?

One obvious possibility is that the rate exhibited is limited by the rate of entry of positively charged species, protons or K⁺ ions, into the inner space of the vesicle. Thus no, or very few, electrons (perhaps only those involved in the initial fast electron entry into cytochrome *a* and Cu_A of the oxidase unit; see below) can pass through the oxidase and hence across the membrane unless there is a simultaneous passage of counterions through the membrane. In this context ionophores would act by increasing this rate so that the enzyme may display its intrinsic catalytic potential. However, if counterion flux through the membrane were the rate-controlling process, it would be difficult to explain why pulsed cytochrome oxidase vesicles catalyse cytochrome *c* oxidation faster than do the resting cytochrome oxidase vesicles, as presumably both would be rate-limited at the same value by the counterion flux through the membrane. It would be even more difficult to account for the fact that the apparent rate constant (2.0 s⁻¹) for pulsed cytochrome oxidase

vesicles in the absence of ionophores is larger than that for the resting cytochrome oxidase vesicles (1.4 s⁻¹) in the presence of ionophores (Table 2). In addition, the rate of permeation of protons through the membrane in the absence of ionophores, at least under the conditions reported in Fig. 4(a), appears too low to support the catalytic rates measured. These considerations seem to exclude the possibility that counterion flux through the vesicle membrane limits the electron-transfer rate to oxygen.

Arguments based on the similar hypothesis that electron transfer is limited by ion flux through the oxidase (i.e. that the enzyme is an ionophore) are also difficult to sustain. It may be proposed that catalytic rates are limited by access of protons to a proton-translocation part of the enzyme; in this case externally added ionophores would act by binding to the oxidase, thereby increasing such access. If this were so, it would be difficult to account for the lack of effect of these ionophores on the activity of the oxidase in free solution (results not shown). Moreover it seems unlikely that molecules such as carbonyl cyanide *p*-trifluoromethoxyphenylhydrazide and nonactin, so different in molecular structure, would bind to the same site with similar effects. However, at this stage we cannot exclude that binding of ionophores to the enzyme may play an important role in rate enhancement in cytochrome oxidase vesicles.

The lack of significant deactivation during the first turnovers despite the clear differences in activity (Fig. 2) leads to an alternative explanation, namely that at the time observation starts a non-equilibrium gradient exists across the membrane. This gradient must influence the rate of transmembrane electron transfer, must be relaxed by ionophores and must be sufficient to exert its full effect. One of the few possibilities open is that this gradient is an electrical potential difference generated by the initial electron-transfer reactions from cytochrome *c* into its oxidase. It is known from kinetic studies (Wilson *et al.*, 1975) that, under the protein concentration and buffer regimes we have employed, electrons are rapidly transferred into cytochrome *a* and Cu_A of the oxidase unit. These rapid redox reactions will necessarily involve an electron transfer into and partially across the membrane, and, although not constituting passage of charge completely across the membrane, would produce an electrical gradient (negative inside), which would oppose subsequent electron transfer. An alternative possibility, the likelihood of which is difficult to ascertain, is that a zero-time gradient may be generated across the membrane of the liposomal vesicle as a result of its formation. On closure the surface charge density due to the lipid polar head groups on the two sides of the membrane will differ, and this may be sufficient to lead to such an electrical potential gradient. Because

of the impermeability of the membrane to positive counterions, this gradient may decay very slowly indeed, i.e. over several hours.

In either case ionophores act to short-circuit such an electrical potential by allowing rapid re-equilibration of positive charges, protons and/or K^+ ions.

The multiturnover experiments, in which the absorbance changes of encapsulated Phenol Red and externally added cytochrome *c* are simultaneously followed, yield additional information. The consumption of internal protons is, under these conditions, much faster than the re-equilibration of protons with the bulk phase. In the absence of ionophores (Fig. 5), the rate of internal alkalization, measured by monitoring the Phenol Red absorbance, follows zero-order kinetics over the whole of the steady-state phase; this indicates, as proposed by Wrigglesworth (1978), that the internal pH does not control the rate. The results depicted in Fig. 5 also show that proton consumption inside the liposomal vesicles is observable even in the presence of nonactin if the overall process is rapid enough (in the case of these experiments by using high concentrations of oxidase, ascorbate and tetramethyl-*p*-phenylenediamine). This is fully consistent with the half-time (approx. 4s) for the collapse of the proton gradient catalysed by non-actin (as measured in Fig. 5b).

A general observation that may be made concerns the correlation, if any, between different conditions that lead to enhancement of the catalytic properties of cytochrome *c* oxidase. Three such factors, discussed in the present paper, are: (i) the effect of phospholipids, (ii) the transition between the resting and the pulsed state of the enzyme and (iii) the effect of ionophores on the vesicle-bound oxidase. As an extreme example, the turnover number of pulsed cytochrome oxidase vesicles in the presence of nonactin is at least 50-fold greater than that of the resting detergent-solubilized enzyme, at the same pH. An intriguing point is that these three factors appear to be largely independent of each other. Thus the transition between resting and pulsed states leads invariably to a 4-fold increase in rate, independent of the environment; similarly, ionophores give a similar enhancement for both functional states of the enzyme. Since these different conditions must ultimately affect the rate-limiting step in the oxidase, this apparent independence is in

some ways unexpected and demands additional clarification.

We are indebted to Dr. S. Villaschi for the valuable collaboration in obtaining electron micrographs of the cytochrome oxidase vesicles and to Professor E. Carafoli and Dr. J. Bonaventura for generously supplying Asolectin samples. An exchange grant from N.A.T.O. (no. 1767) is gratefully acknowledged. M. T. W. acknowledges a grant from the Science Research Council (GR/B5022/5).

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