Respiratory control in cytochrome oxidase vesicles is correlated with the rate of internal electron transfer

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Cytochrome *c* oxidase, after reconstitution into phospholipid vesicles, displays respiratory control. This appears as an inhibition of substrate oxidation (cytochrome *c*) or reduction (O_2) rates which, in the first few turnovers, can be largely removed upon addition of valinomycin, a specific K^+ carrier. We report experiments designed to measure directly the internal electron transfer leading to the reduction of cytochrome a_3/Cu_B in the presence and the absence of a membrane potential. The results suggest that, after the complete oxidation and partial re-reduction of the protein, electron transfer to the binuclear site is valinomycin-sensitive, i.e. is inhibited by the membrane potential. The first-order rate constants calculated in the absence and presence of valinomycin were 0.5–0.6 and 5–6 s^{-1} respectively. Kinetic analysis of the re-reduction process is consistent with the conclusion that the membrane potential is below the critical threshold until the first electron is transferred to the cytochrome a_3/Cu_B site. Furthermore, the respiratory control ratio obtained from the dependence of the internal electron transfer rate constant on valinomycin is always higher (by factor of 2) than that measured under turnover conditions either polarographically or spectrophotometrically. Two possible interpretations of this discrepancy are discussed.

INTRODUCTION

The electrochemical potential across the mitochondrial membrane, which according to Mitchell's chemiosmotic theory [1] drives ATP synthesis, controls the rate of respiration. The mechanism by which the electrochemical potential regulates the redox activity of cytochrome *c* oxidase in mitochondria and in artificial phospholipid vesicles, though extensively studied [2–4], is still unknown. We present here new kinetic experiments which are relevant to this problem.

It is well known that control of respiration in cytochrome oxidase vesicles (COV) and in mitochondria may be removed by compounds (e.g. valinomycin and nigericin) which collapse the electrical and the chemical components of the membrane electrochemical potential gradient [5–7]. On the basis of stopped-flow experiments in which COV and ferrocyanochrome *c* were mixed with oxygen in the presence and absence of ionophores, Brunori *et al.* [8] suggested that the electrical component of the electrochemical potential, rather than ΔpH , inhibits the turnover rate. According to this hypothesis, the valinomycin-sensitive transmembrane electrical potential acts as an allosteric modulator which controls the equilibrium between two functionally different states of the enzyme. Since the turnover number of the enzyme in detergent is limited by internal electron transfer from the electron-accepting sites (cytochrome *a* and/or Cu_A) to the binuclear oxygen-binding site [9], it was postulated that the control is exerted at this level. Recent experiments [10] have indicated that the membrane potential decreases not only the rate of internal electron transfer to the binuclear site, but also the reduction of cytochrome *a* by cytochrome *c* [3,11]. However, a direct experiment to probe the effect of membrane potential on the rate of the internal electron transfer process is still lacking.

We have now investigated the correlation between internal electron transfer to cytochrome a_3/Cu_B and the onset of res-

piratory control by performing, using COV, stopped-flow experiments similar to those carried out with the solubilized enzyme [12]. These experiments clarified the mechanism of the intramolecular reduction of cytochrome a_3/Cu_B , showing that two electrons are transferred internally, albeit with different rates. The essential features of this experiment are shown in Fig. 1; basically, cytochrome *c* oxidase (fully reduced with ascorbate and cytochrome *c*) is mixed with stoichiometric dioxygen in the presence of excess carbon monoxide (see also the Materials and methods section). Within the dead time of the apparatus the protein is oxidized by oxygen and re-reduced by ferrocyanochrome *c* at the level of the electron-accepting pole, cytochrome a/Cu_A (two electron equivalents). These two electrons are then transferred intramolecularly to the binuclear site, cytochrome a_3/Cu_B , on a time scale accessible to observation, while two more electron equivalents of ferrocyanochrome *c* are oxidized. The fully reduced

Fig. 1. Schematic drawing of the species sequentially populated in the stopped-flow experiment designed to follow the internal electron transfer reaction

The box represents a monomer of cytochrome *c* oxidase, containing four redox centres; each dot stands for one electron. Notice that during dead time the protein is fully oxidized and half re-reduced, while 'observation' pertains to re-reduction of the binuclear site. CO, carbon monoxide.

Abbreviations used: COV, cytochrome oxidase vesicles; RCR, respiratory control ratio.

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protein binds carbon monoxide at high rate, given the high concentration of this ligand. Malatesta *et al.* [12] proposed that the first electron transferred intramolecularly to the binuclear site adversely affects the rate of the second transfer (or alternatively that internal electron transfer via parallel independent pathways may occur at different rates). Moreover, the binding of carbon monoxide (employed to drive the equilibrium to the fully reduced state without any turnover) lags behind cytochrome *c* oxidation, implying that reduction of cytochrome *a₃* occurs last. The kinetic model which accounts for the experimental observations with the detergent-solubilized enzyme was fully discussed in [12]. This type of experiment, now carried out using COV, indicates (i) that the same kinetic scheme proposed for the detergent-solubilized protein applies to uncoupled COV, and (ii) that the rate of reduction of cytochrome *a₃* is strongly inhibited by the membrane potential. These new results are discussed in the framework of the mechanism of control of cytochrome oxidase activity by the transmembrane electrochemical gradient.

MATERIALS AND METHODS

Cytochrome *c* oxidase (EC 1.9.3.1) was purified from beef heart according to Yonetani [13], with minor modifications. The concentration of the protein, expressed as the functional unit ($a-a_3$), was determined spectroscopically at 605 nm using the $\Delta\epsilon = 22 \text{ mm}^{-1} \cdot \text{cm}^{-1}$ (reduced minus oxidized) [14]. Type VI cytochrome *c*, type IIS phosphatidylcholine (asolectin) and ionophores were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All reagents were of analytical grade, and were used without further purification.

Stopped-flow measurements were carried out using a Durrum-Gibson stopped-flow apparatus equipped with a thermostatted 2 cm observation chamber. Static spectra were run using an OLIS-converted Cary 14 spectrophotometer (On Line Instrument Systems, Jefferson, GA, U.S.A.). Both the kinetic and the static signals were analysed by a Compaq 286 computer equipped with a mathematical coprocessor. The change in the oxidation state of cytochrome *c* was determined at 563 nm using $\Delta\epsilon = 3.5 \text{ mm}^{-1} \cdot \text{cm}^{-1}$ (reduced minus oxidized). The formation of the carbon monoxide complex of reduced cytochrome *a₃* was monitored at 585 nm. As noted before [12], at 563 nm the signal depends only on the cytochrome *c* absorbance change, while at 585 nm 70% of the observed absorbance change is a result of the formation of the carbon monoxide derivative; thus the time course at 585 nm has been corrected for the contribution of cytochrome *c*.

COV were prepared according to the cholate-dialysis method [5], using 50 mg of asolectin/ml. This procedure was modified, so that: (i) the concentrations of cytochrome oxidase and sodium cholate were increased to 15 μM and 40 mM respectively, with the final lipid/protein concentration ratio being close to 17:1, and (ii) COV were allowed to equilibrate in 0.1 M-K-Hepes, pH 7.8, during the last dialysis step. The fraction of the protein that was mitochondrially oriented (sidedness [15]) was always close to 75%, and the respiratory control ratio (RCR), as determined polarographically [5,7] was 4.9 ± 1.0 (mean $\pm$ s.d.; six different COV preparations).

Rapid mixing experiments were carried out, as for the soluble enzyme [12], by mixing typically 15 μM fully reduced COV in the presence of 100 μM -ferrocyanide *c* and 0.5 mM-sodium ascorbate, with dioxygen (close to 1:1 stoichiometry with cytochrome oxidase) and 1 mM-carbon monoxide. The oxygen concentration was varied in the mixing syringe by adding known amounts of air-equilibrated buffer to the same anaerobic medium. The reduction was achieved directly in the driving syringe of the stopped-flow apparatus; when necessary, ionophores were added

to the COV-containing syringe at this stage, to allow the complete consumption of oxygen before starting the experiment. After reduction, COV were held in the driving syringe of the stopped-flow apparatus for at least 60 min to achieve the complete dissipation of the electrochemical gradient that had built up during the preliminary reduction phase.

RESULTS

Fig. 2 shows the time course of the oxidation of ferrocyanide *c* and the formation of the carbon monoxide complex, as observed at 563 and 585 nm respectively. The experiments were carried out at 7 °C and pH 7.8, because under standard conditions of temperature and pH (20 °C, pH 7.0) the internal electron transfer process was found to be too fast and thus almost undetectable. The oxygen concentration was stoichiometric with the enzyme to allow complete oxidation without additional turnover; this was confirmed by measuring the absorbance change at 563 nm, which indicated the oxidation of cytochrome *c* as expected (see also the Introduction section). The same reaction was also monitored in the presence of valinomycin, nigericin or the two ionophores together.

In the absence of ionophores, the rate of oxidation of cytochrome *c* decreased as a function of time, within approx. 100 ms (Fig. 2a); the apparent first-order rate constant for the

Fig. 2. Time course of oxidation of cytochrome *c* (563 nm) and formation of the carbon monoxide complex (585 nm) in COV.

The reaction is shown in the absence (a) or the presence (b) of valinomycin. Concentrations after mixing: COV, 7 μM ; cytochrome *c*²⁺, 75 μM ; sodium ascorbate, 0.25 mM; O₂, 7 μM ; carbon monoxide, 0.5 mM; valinomycin, 5 μM . Addition of 5 μM -nigericin was without effect. The maximal absorbance changes at 563 and 585 nm (the latter after correction for the contribution of cytochrome *c*) were respectively 0.07 and 0.032.

Table 1. First-order rate constants calculated at 563 and 585 nm in the absence and presence of ionophores

Results are mean values $\pm$ S.D. of the numbers (given in parentheses) of independent experiments carried out using different COV preparations. The superscript ψ indicates the presence of the membrane potential. The concentration of cytochrome *c* was 75 μ M; all other conditions were as in Fig. 2.

Conditions	Rate constant (s^{-1})			
	k_1 , 563 nm	k_2 , 585 nm	k_2^ψ , 563 nm	k_2^ψ , 585 nm
COV	9.9 ± 1.8 (11)	6.4 ± 1.1 (7)	0.6 ± 0.1 (14)	0.5 ± 0.1 (13)
+ Nigericin	10.9 ± 0.9 (3)	7.2 ± 1.1 (3)	0.7 ± 0.1 (3)	0.5 ± 0.2 (3)
+ Valinomycin	10.4 ± 1.1 (7)	6.1 ± 0.6 (7)	—	—
+ Valinomycin + nigericin	10.1 ± 1.7 (7)	6.2 ± 1.7 (7)	—	—

Fig. 3. Cytochrome *c* concentration-dependence of the first-order rate constants observed at 563 and 585 nm

(a) k_1 values of the rapid phase observed at 563 nm in the absence (●) and presence (○) of valinomycin. (b) k_2 values calculated at 585 nm in the presence (□) or absence (■) of valinomycin; for the latter, the rate constant of the fast initial phase is reported. Notice that the value of k_2 at 585 nm corresponds to the rate constant value of the final slow phase observed at 563 nm in the presence of valinomycin (see the text). Also shown are the k_2 values relative to the slow phase observed at both 563 (△) and 585 (▲) nm in the absence of valinomycin. Lines represent the best fit to a hyperbola. All other conditions were as in Fig. 2.

initial phase was 10 s⁻¹, and that of the second phase was 0.6 s⁻¹. A similar trend was observed at 585 nm: the time course showed an initial rapid phase ($k_{app.} = 4$ s⁻¹) followed by a slower one ($k_{app.} = 0.5$ s⁻¹). The initial fast phase at 563 nm (0.04 absorbance

units/2 cm) accounts for the oxidation of 5–6 μ M-cytochrome *c*, i.e. approx. 50–60 % of the total (10 μ M) cytochrome *c* oxidized during the experiment. Since in this experiment the concentration of the mitochondrially-oriented cytochrome oxidase in COV was approx. 5 μ M, the fast kinetic component at 563 nm corresponds to the oxidation of close to one equivalent of cytochrome *c* per functional unit. In agreement with previous observations [12], a very small (often undetectable) increase in absorbance was observed at 605 nm, indicating that, at a high cytochrome *c* concentration, cytochrome *a* and Cu_A are almost completely reduced when observation started (see scheme in Fig. 1).

Addition of nigericin was without effect at both wavelengths (results not shown). On the contrary, addition of valinomycin led to a clear-cut change in the time course of the reaction, in so far as the slow phase ($k_{app.} = 0.5$ – 0.6 s⁻¹), observed at both wavelengths in the absence of ionophores, was no longer present (see Fig. 2). Even in the presence of valinomycin, the time course at 585 nm may not be fitted to a single exponential; after correction for the contribution of cytochrome *c*, the signal at 585 nm often showed a lag phase followed by the reduction of cytochrome *a*₃, similar to what is observed for the enzyme in detergent (extensively discussed by Malatesta *et al.* [12]), and the first order rate constant ($k_{app.}$) was 4–5 s⁻¹. At the end of every experiment, the rate of carbon monoxide binding to fully reduced COV was found to be 30 s⁻¹ at a carbon monoxide concentration of 0.5 mM. As shown and extensively discussed by Antonini *et al.* [10], valinomycin is not acting as a protonophore under our experimental conditions, and thus its only effect is on the electrical component of the electrochemical gradient.

The total absorbance recovery at 563 nm in the presence of the ionophores again accounted for the oxidation of close to two cytochrome *c* equivalents/functional unit in the time range available to observation. It should be recalled (see also the Introduction section) that two electron equivalents of cytochrome *c*²⁺ were oxidized in the dead time of the stopped-flow apparatus.

Table 1 lists the first-order rate constants calculated by fitting the observed transient to a sum of two exponential processes. The rate constants are defined according to the kinetic model scheme given in Fig. 1 and outlined in the Introduction section; thus k_1 and k_2 reflect the first and second electron transfer processes to the binuclear site respectively, under conditions where turnover does not occur. It may be seen that $k_1 = 10$ – 11 s⁻¹ regardless of the presence or absence of valinomycin, while $k_2 = 6$ s⁻¹ in the presence of valinomycin and 0.6 s⁻¹ in its absence.

The dependence of k_1 and k_2 on the cytochrome *c* concentration, between 2- and 20-fold stoichiometric excesses over cytochrome oxidase, has been investigated. The results (Fig. 3) show that in all cases there was a small but measurable dependence of the observed rate constants on the concentration of cytochrome *c*. At 563 nm, in both the presence and the absence of valinomycin, k_1 reached a plateau value of 11 s⁻¹ at cytochrome *c* > 50 μ M (Fig. 3a). By fitting the data to a hyperbola, the apparent Michaelis constant in the presence of valinomycin was 10 μ M, increasing to about 30 μ M in its absence. Within the same range of cytochrome *c* concentrations k_2 showed a plateau at 5–6 s⁻¹, with an apparent K_m of 20 μ M in both the presence and the absence of valinomycin (Fig. 3b). More important is the observation that in the absence of ionophores (Fig. 3b), k_2 displayed a plateau value of 0.6 s⁻¹ which was the same at both 563 and 585 nm; the K_m was about 30 μ M. Every COV preparation has been also characterized in terms of the RCR, as calculated polarographically or spectrophotometrically under turnover conditions. As reported before [7], the RCR determined under turnover conditions by the two techniques is the same.

The average RCR calculated, using six different COV prepar-

ations, from the ratio of the internal electron transfer rate, k_2 , in the absence and in the presence of a membrane potential, was 11.6 ± 1.8 . This value is twice that measured independently under turnover conditions in air (4.9 ± 1.0). Moreover, in this type of experiment the inhibition of enzyme activity is completely prevented by valinomycin, since no significant increase in rate was observed following addition of valinomycin plus nigericin (see Table 1). Results indicating that valinomycin has no significant protonophoric activity under our conditions have been presented by Antonini *et al.* [10].

DISCUSSION

Similar to what was reported for the detergent-solubilized enzyme [12], the rate constant for the transient observed at 585 nm (which monitors the formation of the carbon monoxide complex of reduced cytochrome a_3) is independent of the cytochrome c and carbon monoxide concentrations above a critical value ($> 50 \mu\text{M}$ -cytochrome c and $500 \mu\text{M}$ -carbon monoxide). This result implies that the combination of reduced cytochrome a_3 with carbon monoxide is rate-limited by a monomolecular process, which we assign to one of the two internal electron transfer processes from the electron-accepting sites, cytochrome a and Cu_A , to the binuclear centre. In the presence of ionophores, where any compartmentalization-related effect is collapsed, the kinetic model presented previously for the detergent-soluble enzyme [12] should still be applicable. Our new results fulfil our expectations, since the oxidation of cytochrome c by COV in the presence of valinomycin, which almost completely releases the respiratory control, is biphasic and partly precedes the reduction of cytochrome a_3 . As indicated by the change in absorbance recovery at 563 nm, the faster process ($k_1 = 10\text{--}11 \text{ s}^{-1}$) should correspond to the transfer of a first electron to the binuclear site, while the slower one ($k_2 = 4\text{--}5 \text{ s}^{-1}$) leads to full reduction of cytochrome a_3/Cu_B , and formation of the carbon monoxide complex. The presence of a lag phase at 585 nm, though less clearly detectable than in solution (possibly due to the less favourable optical conditions), confirms that in the absence of a membrane potential the combination with carbon monoxide takes place only after the complete two-electron reduction of the binuclear centre. This finding is in agreement with previous thermodynamic data [16] supporting a co-operative two-electron reduction of the cytochrome a_3/Cu_B sites in the presence of CO. In the absence of valinomycin, as the re-reduction proceeds the internal electron transfer rate becomes inhibited by the increase in membrane potential. The onset of inhibition by the membrane potential gradient is clearly shown by the decrease in the apparent first-order rate constant at 585 nm from $4\text{--}5 \text{ s}^{-1}$ to $0.5\text{--}0.6 \text{ s}^{-1}$ (Fig. 2). The initial oxidation of cytochrome c is only partially affected by addition of valinomycin; therefore at this stage of the reaction, i.e. after complete oxidation of the protein and re-reduction of cytochromes a and Cu_A (both lost during the dead time), the membrane potential is still below a critical threshold value. In terms of the kinetic mechanism outlined above, and at variance with the scheme proposed to occur in solution [12], k_2 is therefore $4\text{--}5 \text{ s}^{-1}$ with fully uncoupled COV, and $0.5\text{--}0.6 \text{ s}^{-1}$ when the membrane potential is controlling the process.

The turnover number, calculated as $4 \times k_2$ to account for stoichiometry [12] was 16 and 2 s^{-1} in the presence and absence respectively of valinomycin (with or without nigericin). The value of 16 s^{-1} is consistent with the turnover rate for cytochrome c oxidation calculated independently for the detergent-solubilized enzyme, taking into account lipid activation (at pH 7.8 and 7°C) [7,12]. Addition of valinomycin leads therefore to nearly 100% release of inhibition; moreover, the presence of nigericin alone

had no effect on the time course. These results confirm that in a time regime corresponding to a few turnovers, the electrical component of the electrochemical potential is responsible for the control of internal electron transfer [8,10]. However, this finding may appear inconsistent with the report that under many turnover conditions the turnover rate increases in the presence of nigericin [2,3]; as extensively discussed elsewhere [10], sensitivity to this ionophore is revealed only at late stages of the steady state, as the vesicle interior becomes more and more alkaline. Although the upper limiting value for k_1 in the presence and absence of valinomycin is the same, the rate constant describing the first internal electron transfer to the binuclear site shows some dependence on the cytochrome c concentration, and the apparent Michaelis constant seems to be significantly higher in the absence of valinomycin. This finding suggests that at this stage, i.e. when observation starts, the membrane potential that has built up, although still insufficient to inhibit electron transfer, may nevertheless produce an electric field (positive outside) which adversely affects the bimolecular reaction between cytochrome c and the oxidase. This is consistent with the findings presented by Gregory & Ferguson-Miller [3], and supported by more recent results from this laboratory [10].

According to the simplest possible mechanism envisaged for this complex system, the following expectation should be verified. Since the turnover of the enzyme is rate-limited by the slowest internal electron transfer process (k_2), and because this is the kinetic step primarily affected by electrochemical potential, the RCR determined under steady-state conditions should be equal to the ratio of the k_2 values in the presence and the absence of valinomycin. Surprisingly, we have observed that the latter RCR value is always larger than the former. At this point we do not have an explanation for this observation. However, it is known that the magnitude of the membrane potential generated by COV depends on a number of physical parameters intrinsic to the vesicle structure [15,18] and on the relative efficiency of charge-separating processes and 'leaks'. The charge-separating processes are synchronous with the redox-linked events (oxygen reduction, proton pumping and electrogenic electron transfer). The structure of the vesicle bilayer modulates in turn the gradient dissipation rate, by affecting the ion permeability coefficients, and the membrane potential breakdown value [19]. Thus one may envisage that, under the conditions herein described, the dissipation of the membrane potential lags behind its generation; as a consequence, the magnitude of the membrane potential created is maximal at the end of the re-reduction of cytochrome a_3/Cu_B , provided that it does not exceed some 160 mV, i.e. the potential breakdown value [19]. Under turnover conditions, i.e. on a time scale of seconds, the ion leak comes into play, delayed at a rate imposed by the intrinsic membrane leakiness and capacitance. Thus, provided that the membrane permeability of COV is low, this may explain why the RCR calculated from the internal electron transfer rate is higher than that measured under steady-state conditions.

A possible alternative accounting for the different RCRs is based on the following consideration. Steady-state activity measurements are always carried out in the presence of excess dioxygen (typically in air), which is known to have a very high rate constant for the reaction with the reduced binuclear site [20] and a large driving force, yielding rapidly and irreversibly the fully oxidized (pulsed) oxidase. On the other hand, the internal electron transfer measurements reported above rely on the binding of CO to drive the equilibrium towards the reduced enzyme, by trapping the binuclear centre in the fully reduced ligand-bound state. Although the equilibrium association constant for CO is high enough ($K = 3 \times 10^6 \text{ M}^{-1}$) [20,21], the rate constant for CO binding ($k = 30 \text{ s}^{-1}$ at 0.5 mM -carbon monoxide)

is not 'infinitely' fast compared with the measured internal electron transfer, and the driving force exerted is definitely lower than in the case of oxygen. Thus it may be possible that in the absence of ionophores, i.e. with a fully developed membrane potential, electron transfer to cytochrome a_3 becomes sufficiently unfavourable to result in an apparent slow-down of the observed rate of CO binding, well below the value expected from the turnover experiments in the presence of oxygen. Thus in the absence of ionophores the thermodynamics of electron transfer to the binuclear centre will play a major role in the control of the observed rate of reduction of cytochrome a_3 . This hypothesis is not inconsistent with the recent observation that energization of submitochondrial particles is associated with a structural change of cytochrome a_3 , corresponding to a high-spin to low-spin transition, as detected by Resonance Raman spectroscopy [22].

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