Control of Cytochrome Oxidase Activity

A TRANSIENT SPECTROSCOPY STUDY

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The kinetics of cytochrome oxidase reconstituted into small phospholipid vesicles (COV) has been followed by transient optical spectroscopy under steady-state and pre-steady-state conditions, in the presence and absence of ionophores. The effect of valinomycin on the activity of reconstituted cytochrome oxidase is shown to depend on the absolute concentration of the ionophore and on the number of turnovers elapsed by the enzyme; this novel observation, which escaped previous investigations, may account for important differences in results and therefore in interpretation of the mechanism of control of the enzyme activity as between Brunori et al. (Brunori, M., Sarti, P., Colosimo, A., Antonini, G., Malatesta, P., Jones, M. G., and Wilson, M. T. (1985) EMBO J. 4, 2365–2368), Gregory and Ferguson-Miller (Gregory, L., and Ferguson-Miller, S. (1989) Biochemistry 28, 2655–2662) and Capitanio et al. (Capitanio, N., De Nitto, E., Villani, G., Capitanio, G., and Papa, S. (1990) Biochemistry 29, 2939–2944). Quantitative analysis of the optical spectra acquired within 10 ms over a large wavelength and time range (500–650 nm and 5 ms to 60 s) under different experimental conditions, indicates that the electrical component of the transmembrane electrochemical gradient controls the rate of the internal electron transfer from cytochrome a-Cu, to cytochrome a3-CuB as well as the cytochrome c to cytochrome a electron transfer. The slow down of cytochrome oxidase activity observed in the presence of valinomycin after several (>10) turnovers is attributed to alkalization of the vesicle interior, which affects the internal electron transfer rate. These two mechanisms of control act most likely independently.

A "cubic scheme," which illustrates the effect of the electrochemical gradient on two states of cytochrome oxidase characterized by different redox and proton pumping activities is presented and discussed.

Mitochondrial cytochrome c oxidase contributes to the proton electrochemical gradient (ΔµH+) driving the synthesis of ATP (1) by virtue of vectorial electron transfer and proton translocation. The collapse of ΔµH+ (which may be achieved by adding a sufficient amount of ionophores) leads to an increase in the rate of oxygen consumption both in mitochondria and in the reconstituted system (cytochrome oxidase vesicles, COV) (2). The respiratory control ratio, i.e., the ratio of the catalytic rates in the presence and absence of ionophores, is between 5 and 20 with cytochrome oxidase reconstituted into small unilamellar vesicles (2, 3). In the absence of ionophores, the rate of oxygen consumption measured in steady-state experiments has been classically considered to be limited by the rate of proton back leak across the lipid bilayer (see Ref. 4). On the basis of rapid mixing experiments, we proposed (5) a different mechanism whereby Δψ, the electrical component of ΔµH+, controls the activity of cytochrome oxidase by shifting the equilibrium between two conformational states of the enzyme (called P and S), characterized by different catalytic rates. Kinetic experiments showed that addition of nigericin (which collapses only ΔpH) had no effect on the rate of cytochrome c oxidation; on the other hand, in the presence of valinomycin (which collapses only Δψ) the catalytic rate was similar to (though smaller than) that observed for the fully uncoupled COV (i.e., in the presence of both valinomycin and nigericin or CCCP). On the basis of these and other results Brunori et al. (5) concluded that Δψ stabilizes the enzyme into a conformational state (S) characterized by a slow turnover rate and lack of proton pumping activity; the latter assumption was introduced into the model because proton pumping has been observed only in the presence of valinomycin and K+ as charge compensating system (1, 5, and references therein). Therefore, a correlation between conformational state of the enzyme, redox activity, and proton pumping seemed interesting and novel and led to the suggestion that cytochrome oxidase, by virtue of its membrane topology (1), behaves in the same way as a "gated channel," which is open and pumping in the more active state, or closed and slipping in the less active one.

Recently, Gregory and Ferguson-Miller (7) and Capitanio et al. (8) measured the steady-state reduction levels of three cytochromes (cytochrome c, a, and a3) and their changes following selective elimination of ΔpH and/or Δψ. These authors failed to observe an increase in the rate of cytochrome c oxidation (or oxygen consumption) upon addition of valinomycin. The 5-fold stimulation of cytochrome oxidase activity which we observed (5) upon addition of valinomycin was attributed by these authors to a contamination of valinomycin or to an excessive H+ leakiness intrinsic to our COV preparation. On the other hand, the same authors observed differences in the steady-state reduction level of cytochrome c and cytochrome a. On the basis of their results, Gregory and

1 The abbreviations used are: COV, cytochrome oxidase vesicles; CCCP, carbonyl cyanide p-chlorophenylhydrazone; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TMPD, N,N,N',N'-tetramethyl-p-phenylenediamine.

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Ferguson-Miller (7) proposed that \( \Delta \Psi \) controls electron transfer from cytochrome c to cytochrome a by decreasing the reduction potential of cytochrome a, while pH affects the internal electron transfer from cytochrome c to cytochrome a, possibly via a conformational change in the enzyme. This model recalls an earlier proposal by McGovern-Moroney et al. (9) in so far as \( \Delta \Psi \) and \( \Delta \phi \) induce separate effects on cytochrome oxidase. Their general conclusions were supported by the results of Maisen-Peteri and Malmström (10), while Capitano et al. (8) suggested that \( \Delta \Psi \) controls electron flow not only from cytochrome c to cytochrome a but also from the latter to cytochrome a and finally from the binuclear site to oxygen.

In this paper we report an extensive reinvestigation of the problem. The new data resolve some of the experimental discrepancies and show that our previous results (5) cannot be attributed to ion-leaky vesicles. The results obtained by rapid transient spectroscopy show that \( \Delta \Psi \) decreases the rate of both internal and external electron transfer in cytochrome oxidase, while we have no evidence suggesting an inhibition of the reaction of the binuclear center with oxygen. An alkalization of the vesicle interior (and not \( \Delta \phi \)) observed after many (>10) turnovers is proposed to inhibit the internal electron transfer rate as observed after many (>10) turnovers, in agreement with direct kinetic experiments carried out with the enzyme in solution (12, 13).

A model for control of proton pumping and redox activity of cytochrome oxidase is presented following the idea that the linkage between ligand binding and mechanical work of a pump can be described by a cubic scheme (14, 15). Malmström (16–18) elaborated a model for proton pumping by cytochrome oxidase based on the assumption that the enzyme may exist in two conformational states, called \( E_1 \) and \( E_2 \). In this paper we discuss our data within the framework of such a “cubic scheme”, which accounts in general terms for the complex relationships between the activities of the enzyme and the transmembrane electrochemical potential gradient.

### MATERIALS AND METHODS

Cytochrome c oxidase was purified from ox heart according to Yonetani (19). The final pellet was dissolved in 0.1 M sodium potassium phosphate pH 7.4, containing 0.5% Tween 20, at a protein concentration of approximately 0.50 mM. Samples were frozen in liquid nitrogen and stored at -70°C. The protein was used within 2 months from preparation.

Protein concentration was determined spectrophotometrically using a 0.5x (reduced-oxidized, at 605 nm) of 22 mM^-1 cm^-1/functional unit (a+a).

Soybean phospholipids (L-alpha-phosphatidylcholine type II S from Sigma) were purified by acetone-ether fractionation cycles. Reconstitution of COV was obtained by the cholate dialysis method (20). The buffer inside the vesicles was 0.1 M potassium HEPES, pH 7.3. The buffer outside the vesicles was 10 mM potassium HEPES containing 384 mM KCl and 49.8 mM sucrose, pH 7.3 (ionic strength about 50 mM). The phospholipid to protein weight ratio was 50:1.

The orientation of cytochrome oxidase in the reconstituted system was about 85% of the molecules with cytochrome a facing outside. 8 or 16 µM cytochrome c, 10 mM sodium ascorbate, and 0.1 or 0.2 mM TMPD, with or without ionophores (1-10 µM).

The apparatus used was a rapid-scanning photodiode array spectrofluorometer (Tracor Northern 6500, Tracor) adapted to a Gibson-Durrum stopped-flow apparatus. The absorption spectra were recorded at 10 ms/spectrum (1024 data points/spectrum) over a 150-nm wavelength range (500-650 nm). Up to 64 spectra were collected at different times after mixing. TN 6500 data sets were transferred to a Micro VAX 3500 for fitting of the observed time courses at different wavelengths using FACSIMILE program which carries out the simultaneous fitting of several observed time courses into reaction schemes using numerical integration of the ordinary differential equations.

The kinetic scheme employed for fitting the data of Fig. 4 was as follows:

\[
\begin{align*}
\tau^+ &\rightarrow \tau^- \\
\tau^+ + \text{OOO} &\rightarrow \tau^+ + \text{IOOO} \\
\text{IOO} &\rightarrow \text{OII} \\
\tau^+ + \text{OII} &\rightarrow \tau^+ + \text{IOI} \\
\text{IOI} &\rightarrow \text{IIII} \\
\text{III} + O_2 &\rightarrow \text{OOOO} \\
\end{align*}
\]

**SCHEME 1**

The digits (0 and 1) designate, according to Malmström (17), the four redox centers of cytochrome oxidase in the order (from left to right): cytochrome a, CuA, CuB, cytochrome a, O representing an oxidized metal and I a reduced one. For each kinetic step the forward and reverse reaction rates are given in parentheses. The inhibition of the turnover rate of reconstituted cytochrome oxidase observed in the presence of valinomycin (see “Results”) was fitted assuming that the internal electron transfer step is slowed down exponentially (with rate \( k_{e+} \)) as the reaction proceeds, providing a phenomenological description of the process. The following 3 extinction coefficients were used (mm^-1 cm^-1) according to Brunori et al. (25): 685 nm, reduced-minus-pulsed (III-0000) = 19; half-reduced-minus-pulsed (II0) = 15.2; cytochrome c^+cytochrome c^+ = 0.86-550 nm, cytochrome c^+cytochrome c^+ = 19.2; reduced-minus-oxidized (III-0000) = 1.

**RESULTS**

The stopped-flow experiments carried out in the present investigation have been conceived to (a) evaluate current discrepancies between our laboratory and others with reference to the effect of valinomycin on the turnover rate of cytochrome oxidase reconstituted in phospholipid vesicles, (b) assess the role of each component of the membrane proton electrochemical gradient on the rates of electron transfer between cytochrome c and cytochrome oxidase and/or within cytochrome oxidase, and (c) unify the available experimental evidence into a general model.

Fig. 1 shows the time course of cytochrome c oxidation in air by COV, at two cytochrome c (10 and 100 µM) and COV (0.05 and 0.5 µM functional units) concentrations, all after mixing. Ionophores were always added to the COV-containing syringe in the following amounts: either valinomycin at different concentrations (0, 1.0, and 10 µM, traces 1–3, respectively, in Fig. 1) or 10 µM valinomycin and 10 µM CCCP (traces 4 in Fig. 1). In all the experiments depicted in Fig. 1 the final dioxygen concentration (~270 µM) was more than enough to allow for complete oxidation of the cytochrome c.
Moreover, on comparing the time courses in panels A and C of Fig. 1 it may be recorded that although the number of enzyme turnovers is the same, the effect of valinomycin is in the two experiments slightly different, and specifically more potent under conditions were the COV concentration is smaller.

In panel B of Fig. 1, in which the total cytochrome c concentration is 10-fold lower than in panel A (five turnovers), the catalytic rate in the presence of valinomycin is, however, more similar to the maximal rate observed with fully uncoupled COV. The conditions and the results reported in panel B are quite similar to those of our previous paper (5); on the other hand panel C shows results obtained under conditions similar to those used by Gregory and Ferguson-Miller (7) and Capitanio et al. (8). Therefore, provided there is sufficient dioxygen available, depending on the total concentration of valinomycin, COV (lipid), and cytochrome c (yielding different numbers of turnovers), the effect of the ionophore is different. This is the basis for the varying observations and apparent discrepancies previously noted.

To further clarify the observations depicted in Fig. 1, additional experiments were carried out by diluting COV pretreated with valinomycin. The results of this experiment are given in Fig. 2. The top panel shows an experiment similar to that of Fig. 1B; treatment of coupled COV (top trace) with 10 \( \mu \text{M} \) valinomycin (middle trace) releases about 80% of the RCR, as determined following further addition of 10 \( \mu \text{M} \) CCCP.
(bottom trace). Either coupled or valinomycin-treated COV where then diluted 100-fold with isotonic buffer and the cytochrome c oxidation assay repeated (Fig. 2, panel B). Following dilution, the absolute COV and valinomycin concentrations were 1/100th of the initial value (see figure legend for details); as a consequence the time courses clearly indicate that, under these conditions, valinomycin is no longer capable of dissipating the membrane potential, since the observed rate constant for cytochrome c oxidation is close to that measured in the absence of the ionophore or in the presence of an amount of ionophore identical to that obtained following dilution of the ionophore pretreated sample (see Fig. 2). Moreover, further addition of excess valinomycin to the untreated or pretreated COV sample restored the effect as described in Fig. 1. The loss of respiratory control upon dilution of the valinomycin pretreated sample is most likely due to dissociation of the ionophore from the vesicle membrane into the aqueous phase. We conclude that although the valinomycin lipid/water partition ratio greatly favors the membrane, 100-fold dilution decreases the amount of lipid available for dissolving apolar compounds. The results shown in Fig. 2 may be interpreted on the basis of a simple model shown below in Equation 1.

\[ C + n \text{V} \rightleftharpoons U \quad K = \frac{[U]}{[C][V]^n} \]  

where \( C \) represents coupled COV, \( U \) is COV containing \( n \) molecules of valinomycin, \( V \) is free aqueous valinomycin, and \( K \) is the valinomycin isothermal lipid/water partition equilibrium constant. Given the mass conservation, which requires that \( C_0 = [C]+[U] \) and \( V_0 = [V] + n [U] \) (where \( C_0 \) and \( V_0 \) indicate the total COV and valinomycin concentrations), and assuming that \( n = 1 \) molecules of valinomycin partition into the phospholipid membrane, it is possible to calculate valinomycin-free COV concentration.

\[ \frac{[C]}{C_0} = \frac{[V_0] - [C_0] + 1}{[V_0] - [C_0]^2 + 4K[C_0]^n} \]  

This equation allows the calculation of the fraction \( f_U \) of uncoupled COV (i.e. containing 1 molecule of valinomycin/vesicle) and the fraction of valinomycin in COV \( f_V \), at constant temperature and any given COV and valinomycin concentration:

\[ f_U = \frac{C_0 - [C]}{C_0} \quad f_V = \frac{C_0 - [C]}{V_0} \]  

The results of these calculations are given in Table I for a value of \( K = 10^6 \text{ M}^{-1} \). For example by diluting 1 \( \mu \text{M} \) COV containing 10 \( \mu \text{M} \) valinomycin by a factor of 100, the fraction of uncoupled COV decreases 10-fold (compare samples 3 and 4 in Table I), due to dissociation of the ionophore into the aqueous medium. Therefore, it accounts for the lack of effect of valinomycin in dissipating the membrane potential under these conditions.

From the above-mentioned experiments, it is clear that when several turnovers have been carried out by COV, the activity of valinomycin in dissipating the membrane potential is apparently decreased. Again, this failure in efficiency is only apparent since at early stages of the steady-state experiment (Fig. 1A, inset) or when the total number of enzyme turnovers is small (Fig. 1B) there is no inhibition.

To clarify the nature of the onset of the observed inhibition, which is the basis of the reported controversy, we have carried out steady-state experiments in which, however, ferrocytochrome c, is continuously regenerated by ascorbate and TMPD. Therefore it is possible to investigate the effect of valinomycin at early (low number of turnovers) and late stages (high number of turnovers) of the steady-state. The experiments, which are shown in Figs. 3 and 4 were carried out by acquisition of complete optical spectrum as a function of determined time values (see "Materials and Methods").

![Fig. 3. Time-resolved pre-steady and steady-state difference spectra of cytochrome c and cytochrome oxidase.](image)

60 spectra were recorded at different times (from 4 ms to 160 s) after mixing a buffer containing 134 \( \mu \text{M} \) oxygen with a degassed solution containing 8 \( \mu \text{M} \) cytochrome c and 3 \( \mu \text{M} \) COV supplemented with 10 \( \mu \text{M} \) Na+/ascorbate and 0.1 \( \mu \text{M} \) TMPD in the presence and absence of ionophores. The buffer was 10 \( \mu \text{M} \) K+/HEPES, pH 7.3, 43.6 mM KCl, 46.1 mM Sucrose, and temperature 20 °C. The data are represented as difference spectra relative to the corresponding fully oxidized spectrum to compensate for scattering. The 60 spectra are superimposed on the left and plotted sequentially as a function of log time on the right. a, no ionophores added; b, valinomycin 5 \( \mu \text{M} \); c, valinomycin 5 \( \mu \text{M} \) and CCCP 5 \( \mu \text{M} \) (all after mixing).

<table>
<thead>
<tr>
<th>Sample</th>
<th>( C_0 )</th>
<th>( V_0 )</th>
<th>( f_U )</th>
<th>( f_V )</th>
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<td>1</td>
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<td>38.2</td>
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<td>9.9</td>
</tr>
<tr>
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<td>0.1</td>
<td>9.0</td>
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</tr>
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<td>6</td>
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5 mM valinomycin; and (c) COV + 5 mM valinomycin and 5 mM CCCP. Addition of nigericin (5 μM) to coupled COV is without effect and yielded difference spectra identical to those shown in Fig. 3A (data not shown). Similar experiments were carried out at different concentrations of cytochrome c (from 4 to 8 μM), TMPD (from 0.05 to 0.1 mM), and oxygen (from 87 to 135 μM) all after mixing. Qualitative inspection of the data shown in Fig. 3 clearly indicates that the steady-state reduction level of cytochrome c is higher in the completely coupled than in the uncoupled state (panels A and C). This is a straightforward indication that the steady-state rate of oxidation of ferrocytochrome c, and therefore the rate-limiting step of the cytochrome oxidase reaction are enhanced following addition of the complete set of ionophores. It is also very interesting to note that in the presence of nigericin alone (Fig. 3B) the steady-state redox level of cytochrome c and cytochrome a are time-dependent. At early times, where few turnovers have been completed by COV, the steady-state level of cytochrome c is virtually identical to that obtained with the fully uncoupled state. At later times (more turnovers have been completed), however, there is a significant increase in the reduction level of cytochrome c, which approaches that obtained in the absence of ionophores. Although less evident, the same phenomenon occurs at wavelengths where the cytochrome a contribution is dominant. Under these conditions the efficiency of electron transfer changes as a function of time and this transition must be assigned to a change in the rate constant or in the “location” of the rate-limiting step.

In all cases, the time-resolved spectra, analyzed as described under “Materials and Methods,” yield quantitative information on the reduction level of the different species at all times ab initio to the end of the reaction, i.e. from the initially fully reduced to the final fully reduced state via the partially reduced steady-state.

Fig. 4 depicts one presentation of the overall time course in terms of reduction level of cytochrome c and cytochrome a (top and bottom panels, respectively) under the conditions of Fig. 3. Analysis of the spectra acquired synchronously (i.e. within 10 ms) at all the different wavelengths, provides quantitative information on the steady-state reduction level of the two cytochromes and on the length of the steady-state phase (which in turn yields information on the efficiency of catalysis), under the different experimental conditions (oxygen, TMPD, cytochrome c, and ionophore concentrations). Within the errors of the spectral deconvolution, at steady-state, cytochrome a was always found to be fully oxidized and TMPD and CCCP always fully reduced. Moreover, both CuA and CuB do not contribute to the extinction changes in the wavelength range covered by these experiments. The solid lines in Fig. 4 are the best fits obtained by fitting the data points to a model which will be described under “Discussion.” Given the concentrations of the reagents, the reaction of the fully reduced enzyme with oxygen (leading to the pulsed enzyme (26)) and the subsequent initial reduction of cytochrome a and CuA by reduced cytochrome c are very fast (thus largely lost in the dead time of the apparatus) (27). During the pre-steady-state phase of the time course (up to ~0.25 s on the time scale of Fig. 4, see the left panels), the fraction of reduced cytochrome c decreases; a small decrease in the reduction level of cytochrome a synchronous to that of cytochrome c is also detected.

During the steady-state (starting from about 0.5 s on the time scale of Fig. 4, see left panels) the reduction level of cytochrome a is about 30% and that of cytochrome c about 50% with coupled COV (and also in the presence of nigericin).
With fully uncoupled COV (i.e. the presence of valinomycin plus nigericin or valinomycin plus CCCP), the steady-state reduction level of cytochrome a is increased (40%), while that of cytochrome c drops from 50 to 30% reduction. Given that the rate of cytochrome c reduction by ascorbate and TMPD is unaffected by the presence of ionophores (as obtained from the fit), the change in the oxidation level of cytochrome c is consistent with the enhanced cytochrome oxidase activity. Although the steady-state level of cytochrome a is about the same in the case of coupled and fully uncoupled COV, the turnover rates are very different, as demonstrated also by the different steady-state lengths (see below). Consequently, the cytochrome a electron input and output rates must both be equally accelerated by the collapse of the electrochemical gradient.

In the presence of valinomycin, the peculiar phenomenon already described of a progressive change in the steady-state reduction level of cytochrome a and cytochrome c was observed (see Figs. 3 and 4). During the initial turnover phase (up to 1-2 s), the reduction levels of cytochrome c and cytochrome a are both similar to those observed for fully uncoupled COV, indicating equally enhanced activity. Subsequently, however, the steady-state level of both cytochrome a and cytochrome c begin to drift toward a higher reduction level, approaching a new apparent steady-state from 8-10 s on. The concerted increase in the level of reduction of cytochrome a and cytochrome c indicates that in the presence of valinomycin alone the activity of the enzyme is progressively inhibited as the reaction proceeds. We explain this observation with an inhibition of the internal electron transfer rate induced by an increase of pH inside the vesicles, occurring after several turnovers and resulting from internal proton consumption because of the scalar and vectorial process (the latter enhanced by the effect of valinomycin). This interpretation is consistent with the results obtained by stopped-flow using COV with phenol red inside (11) and with the enzyme in solution (12, 13), which show that an increase in pH decreases considerably the internal electron transfer from cytochrome a-CuA to the cytochrome a5-CuB.

The phenomenon of a progressive shift in the steady-state level in the presence of valinomycin was also observed in the fully coupled COV when the experiment was carried out using higher concentrations of cytochrome c, TMPD, and oxygen (data not shown). A change in the steady-state level of both cytochrome c and cytochrome a, analogous to that depicted in Fig. 4B but obtained in the absence of ionophores, indicates that the alkalization of the vesicle interior slows down COV activity also in the presence of a fully developed proton electrochemical gradient.

Once more we wish to stress that looking carefully at some of the traces of Fig. 1. (inset to panels A obtained in the presence of valinomycin) the same slow-down of activity can be observed. The apparent rate constant measured during the presence of valinomycin is similar to that obtained in the presence of valinomycin plus CCCP; after several turnovers, the observed rate constant decreases and tends toward that observed in the absence of ionophores (see above). This is a clear indication that in the presence of valinomycin the reaction rate after the initial turnovers decreases to a value more similar to that measured with fully coupled COV. We believe that Gregory and Ferguson-Miller (7) and Capitanio et al. (8) did not observe these effects because of technical reasons (manual mixing (7) or slow response of the O2 electrode relative to a stopped-flow apparatus (8)). Thus, their analysis of the steady-state levels of the cytochromes was carried out when the onset of inhibition was already established. Moreover, analysis of the overall time course (see also Ref. 5) indicates that the control exerted by Dψ is developed after approximately one turnover, while inhibition caused by alkalization of the vesicle interior requires many turnovers. As a matter of fact, the largest effect in COV activity induced by valinomycin in both types of experiments (in Figs. 1 and 3) can be seen only within 2-3 s from the initial time of observation i.e. up to approximately 5-10 turnovers, since afterward internal alkalization begins to exert its effect.

Finally, the lengths of the steady-state phases provide further support to the findings illustrated above. The steady-state length is longer for coupled vesicles or in the presence of nigericin (>40 s) intermediate in the presence of valinomycin alone (35 s), and shortest in the case of fully uncoupled COV (25 s), at any given oxygen concentration (e.g. 87 μM in the experiment of Fig. 2).

**DISCUSSION**

We first discuss the new experimental results reported here in comparison with similar data published by us and others (5, 7-10) in order to clarify the relative role exerted by Dψ and ΔpH in the control of cytochrome oxidase activity. Second, we present a kinetic model, based on that of Wilson et al. (28), capable of describing in some detail the kinetics of cytochrome c oxidation in COV. Finally, we present and describe a cubic scheme for proton pumps which accounts for the linkage between electron transfer, proton pumping, and membrane potential in cytochrome oxidase reconstituted into small unilamellar vesicles.

**Effect of Valinomycin—**As discussed in the Introduction, the effect of valinomycin on the rate of cytochrome c oxidation by COV has been under question. Our previous results (5) indicated that the membrane potential is the major factor governing the efficiency of electron transfer and proton pumping in COV. The basis for this statement is the enhancement of the electron transfer activity by valinomycin (an electrogenic K+/H+ uniporter, which dissipates the membrane potential) and the lack of effect of nigericin (an electroneutral K+/H+ antiporter which collapses proton chemical gradients) in COV extrinsically impermeable to protons (i.e. high RCR vesicles in which the electrogenic rate of proton import is low, yet the electron transfer rate is higher, see Brunori et al. (5)).

The new experiments reported here implement previous data in terms of the fundamental parameters which appear to control the efficiency of catalysis in the reconstituted system: (a) the concentration of substrates (cytochrome c, O2, and internal and external pH); (b) the concentration of COV (strictly speaking the concentration of vesicles containing oxidase molecules); (c) the proton permeability of the vesicle membrane; (d) the geometry and topology of the system; and (e) the type and specific amount (relative to lipid) of ionophore.

The experiments reported in Fig. 1 and commented above, demonstrate that the difference in the effect of valinomycin on COV activity reported by us (5) on one hand, and by Gregory and Ferguson-Miller (7) and Capitanio et al. (8) on the other, reflects intrinsic properties of the system. Contrary to what suggested by Gregory and Ferguson-Miller (7), our data cannot be explained by an enhanced proton permeability of our COV preparation or a protonophoric activity of our valinomycin batches because of the following. (i) A high rate of passive (membrane-potential-driven) proton back-leak is excluded because it should be more evident in the experiments involving 50 turnovers (Fig. 1, panels A and C), given the large proton gradient built up and the slow overall time course (t1/2 10 s); on the contrary, maximal stimulation of COV...
activity by valinomycin is observed in panel B of Fig. 1 where only five turnovers are carried out, and complete oxidation of cytochrome c occurs with a half-time of 0.6. (ii) A possible contamination or a protonophoric activity of valinomycin is also excluded on the time scale of the experiments reported above because with different valinomycin to phospholipid ratios (Fig. 1, panels A and C and see also Table I) a similar effect on COV activity is seen. (iii) Finally, Sarti et al. (29) have shown that, in the presence of valinomycin, proton pumping synchronous to cytochrome c oxidation is observed with our COV, proving that they are impermeable to protons on a time scale of several seconds. We conclude therefore that collapse of $\Delta \psi$ leads to a large increase in the redox activity of COV, and failure to observe this effect is related to the experimental conditions employed. We believe that other authors (7, 8) failed to observe an increase in the rate of ferrocyanochrome c oxidation (or oxygen consumption) upon addition of valinomycin alone because they carried out many turnover experiments and at relatively low concentration of COV and/or valinomycin.

**Analysis of Transient Spectra**—This indicates that two kinetic steps are under the control of the electrical component of the gradient (as seen from the parameters in Table II). This conclusion is based on the steady-state reduction levels of the system components and the rate parameters obtained by fitting before the inhibitory effect (attributed to alkalization of the vesicle interior) comes into play. The rate of cytochrome c reduction by the excess ascorbate and TMPD is found independent of ionophores (see Table II). The selective collapse of $\Delta \psi$ by valinomycin leads to a more oxidized steady-state level of cytochrome c, which is fully consistent with the release of respiratory control. Because the increased oxidase activity is associated with a nearly constant level of reduction of cytochrome a, it is concluded that the rates of electron input and output to and from cytochrome a are both increased by valinomycin, a finding which may be just coincidental or mechanistically important. Therefore, the electrical component of the membrane potential exerts a control on cytochrome oxidase at two different levels, i.e. on the external electron transfer from cytochrome c to cytochrome $a_{-}Cu_{a}$, in agreement with Gregory and Ferguson-Miller (7), possibly by a thermodynamic mechanism and on the internal electron transfer to the binuclear center, as proposed originally by us (5). This conclusion is supported by other experimental data. Thus, we reported (30) that the intramolecular electron transfer from cytochrome $a_{-}Cu_{a}$ to the binuclear center in COV, measured using ruthenium hexamine (II) as a reductant, is sensitive to the proton electrochemical gradient under conditions in which no effects related to alkalization of the vesicle interior are possible (given the limited turnover). Moreover, we have followed by stopped-flow experiments the pH dependence of the internal electron transfer rate constant(s) and reported elsewhere (13, 30) full evidence that in solution and in COV in the presence of valinomycin, the rate-limiting step in turnover is indeed the rate of reduction of the binuclear site.

After several (>10) turnovers, alkalization of the vesicle interior begins to exert its effect on the internal electron transfer rate. This interpretation is consistent with the results reported by Sarti et al. (31) and Wrigglesworth (32) in which the internal vesicle pH was monitored directly during turnover using phenol red. As shown in Figs. 3 and 4, in the presence of valinomycin both the steady-state reduction levels of cytochrome c and cytochrome a increase at longer times. This type of effect, typical of the system and the geometry of COV, was previously reported by other authors, and attributed either to the membrane $\Delta \phi$ (9, 10) or to internal alkalization (7, 8). It is noteworthy to add that we have observed a similar inhibition of COV activity after many turnovers even in the absence of ionophores, i.e. when the proton electrochemical gradient is fully developed. And, therefore, we conclude that alkalization of the vesicle interior, and not $\Delta \phi$, controls the cytochrome $a_{-}Cu_{a}$ to cytochrome $a_{3}Cu_{a}$ electron transfer (probably acting independently from the control exerted by $\Delta \phi$). Under conditions similar to ours, Gregory and Ferguson-Miller (7) reported that addition of valinomycin alone is associated with an increase in the reduction level of cytochrome a, with no change in the cytochrome c reduction level. This observation was their motivation to conclude that addition of valinomycin enhances electron transfer from cytochrome c to cytochrome a, with no effect on the internal electron transfer process. On the contrary, we have observed that in the presence of valinomycin the reduction level of both cytochrome a and cytochrome c change synchronously (Figs. 3 and 4). We therefore argue that the result reported by Gregory and Ferguson-Miller (7) was biased by the nonsimultaneous acquisition of the spectral data at all wavelengths and lack of the time resolution of their spectrometer, given that in the presence of valinomycin steady-state levels are time-dependent. Within the time interval corresponding to the initial turnover phase, collapse of the electrical component of the gradient leads to a steady-state level (and a catalytic efficiency) equal to that achieved with totally uncoupled COV.

Capitanio et al. (8) reported that $\Delta \phi$ controls also the cytochrome $a_{3}$ to dioxygen electron transfer, since following addition of ionophores (valinomycin plus nigericin) a significant amount (10%) of cytochrome $a_{3}$ was reduced also in the presence of a large excess oxygen. This finding is somewhat surprising given that the reaction of reduced cytochrome oxidase with oxygen is indeed very fast both for soluble oxidase and for COV (32-34). We have no direct data to exclude such a possibility, although our analysis of the complete optical spectra is consistent with a fully oxidized cytochrome $a_{3}$ during steady-state. We may, however, point out that spectral changes related (for example) to a pulsed-to-resting transition (28) may complicate the interpretation of slow and small absorbance changes observed at a single wavelength.

The solid lines through the data points in Fig. 4 were obtained by fitting experimental data to a kinetic model. Both

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**Table II**

Set of rate constants obtained by fitting the data in Fig. 4 to the kinetic model

<table>
<thead>
<tr>
<th>Rate constant</th>
<th>Control</th>
<th>Plus Valinomycin</th>
<th>Plus Val+Cytochrome</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_{1}$ (s$^{-1}$)</td>
<td>3.6</td>
<td>3.6</td>
<td>3.6 (25)</td>
<td></td>
</tr>
<tr>
<td>$k_{2}$ (M$^{-1}$ s$^{-1}$)</td>
<td>$1.09 \times 10^{7}$</td>
<td>$3.14 \times 10^{7}$</td>
<td>$3.14 \times 10^{7}$ (37, 38)</td>
<td></td>
</tr>
<tr>
<td>$k_{3}$</td>
<td>$2.14 \times 10^{7}$</td>
<td>$1.06 \times 10^{7}$</td>
<td>$1.06 \times 10^{7}$ (35, 36)</td>
<td></td>
</tr>
<tr>
<td>$k_{4}$</td>
<td>4,000</td>
<td>4,000</td>
<td>4,000 (35, 36)</td>
<td></td>
</tr>
<tr>
<td>$k_{5}$</td>
<td>4,000</td>
<td>18,000</td>
<td>18,000</td>
<td></td>
</tr>
<tr>
<td>$k_{6}$ (s$^{-1}$)</td>
<td>8.3</td>
<td>150 to 4.6</td>
<td>150 (30)</td>
<td></td>
</tr>
<tr>
<td>$k_{7}$</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>$k_{8}$ (M$^{-1}$ s$^{-1}$)</td>
<td>$1 \times 10^{6}$</td>
<td>$1 \times 10^{8}$</td>
<td>$1 \times 10^{8}$ (32)</td>
<td></td>
</tr>
<tr>
<td>$k_{9}$ (s$^{-1}$)</td>
<td>0.24</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Control of Cytochrome Oxidase

The fitting procedure and model, based on Wilson et al. (28) and Brunori et al. (25), are outlined under “Materials and Methods.” Best fits were obtained by repetitive numerical integration of the differential equations which describe the kinetic mechanism and comparison of the computed and experimental data. We discuss here only some novel features of this model. The first feature is that four consecutive electron transfer steps from ferrocytochrome c to the oxidase are included making the mechanism more complex albeit more realistic. Secondly, it is assumed that the initial electron acceptor is cytochrome a and that a very fast electron relaxation with Cu₆₅ follows (35, 36). Finally, the onset of inhibition which is found in the presence of valinomycin (extensively discussed in this paper) is introduced. Its simulation, however, is on a purely phenomenological basis. It is assumed that the rate-limiting step of the catalytic cycle, i.e. the internal electron transfer rate to the oxygen-binding site (\(k_t\)) is exponentially damped with a time constant (\(k_{\text{damp}}\)) which was obtained by the fitting procedure (see model). This phenomenological approach has the advantage of postponing (to further work) the classification of the event responsible for the onset of inhibition. Table II shows the values of the parameters of the fit, which indicate consistency with previously reported data (see references therein). It is interesting to note the following.

1) The rate constant for reduction of cytochrome c by ascorbate and TMPD is unaffected by the presence of an electrochemical potential gradient.

2) The equilibrium between cytochrome c and cytochrome a is perturbed by the development of a membrane potential (as found also by Gregory and Ferguson-Miller (7)). The ratio (\(k_a/k_c\)) increases from 0.5 to about 3.0 when valinomycin is added to coupled COV, the latter value being very close to that obtained with cytochrome oxidase in detergent (37, 38). It follows that the \(E_a\) of cytochrome a is increased by some 45 mV upon removal of the electrical gradient, a value to be compared with -75 mV as obtained by Gregory and Ferguson-Miller (7). Both findings are in agreement with the observed changes in steady-state reduction level of cytochrome c reported here (see Figs. 3 and 4).

3) The internal electron transfer process (\(k_i\)) is essentially irreversible (i.e. \(k_i \approx 0\)) under the conditions employed (high dioxygen), and (more important) the process appears highly electrogenic since \(k_{\text{damp}}\) drops from 130 to 8 s⁻¹ following development of the membrane potential (see also Ref. 30). Thus, the quantitative analysis of the transient spectra shows that the effect of \(\Delta \psi\) is both external and internal to cytochrome oxidase.

A Cubic Model—we have previously proposed (5) that the electrochemical potential across the membrane controls the equilibrium between two conformational states of cytochrome oxidase, which were designated as P (pumping) and S (slipping). The two-state voltage-gated model envisages that the transition from the P to the S state, induced by \(\Delta \psi\), is associated to inhibition of the turnover number and loss of proton pumping. A quantitative treatment of the effect of \(\Delta \psi\) on the internal electron transfer rate was published by Malvesta et al. (39). Here we present a correlation of this two-state model with other models, in an attempt to rationalize all available data within a cubic scheme for a molecular pump, as proposed by Wyman (14) and first applied to cytochrome oxidase by Wikström and Krab (15). This postulates that the reaction of the enzyme with two ligands (in this case electron and proton) leads to pumping when their binding and release is coupled to a conformational transition of the macromolecule from an input to an output state (relative to the membrane). On the basis of kinetic data, Malmström (16-18) suggested the input and output conformations to be designated as \(E_1\) and \(E_0\), respectively. Both states are populated during turnover under pumping conditions, and the rate of the conformational transition \(E_1 \rightarrow E_0\) is limiting electron transfer to the binuclear site. Prominent differences between \(E_1\) and \(E_0\) are (i) the rates (and the thermodynamics) of electron transfer external and internal to the enzyme; (ii) the structure of the binuclear center which is more “open” in \(E_0\); and (iii) the side of the membrane where uptake and release of protons occurs (the matrix or the cytosol).

A cubic scheme, illustrating the transitions between \(E_1\) and \(E_0\), is shown in Fig. 5. This is an extended allosteric model with the novelty that the electrical component of the gradient across the membrane is the primary effector controlling the rates of the conformational changes, and thereby the relative population of the two allosteric states at steady-state. Let us now examine one complete cycle of the enzyme, bearing in mind that only some of the states (redox and conformational) will be significantly populated at steady-state, and that both \(E_1\) and \(E_0\) are fully competent in carrying out a complete redox cycle. To accomodate the overall stoichiometry of the reaction under conditions in which the proton pump is operative, the fully oxidized enzyme (0000) in the \(E_1\) state is partially reduced from the C side and protonated (from the M side), with a stoichiometry of 2e⁻ and 4H⁺. According to Brzezinski and Malmström (40) and Gray and Malmström (41), the internal electron transfer to the binuclear center is rate limited by the conformational transition \(E_1(1100) \rightarrow E_0(1100)\) with subsequent very rapid formation of \(E_1(0011)\) and binding of \(O_2\) to the reduced binuclear center. This half-cycle leads to species \(E_1(0011-O_2)\), which is the peroxo intermediate observed even at room temperature by flow-flash experiments.

FIG. 5. Cubic scheme illustrating the proton pumping and activity control in cytochrome oxidase. The two lateral faces \(E_1\) and \(E_2\) correspond to two different conformational states of cytochrome oxidase. The digits in parentheses designate (according to Malmström, 17) the four redox centers in the order (from left to right): cytochrome a, Cu₆₅, Cu₉₅, and cytochrome a₇₅. 0 representing an oxidized metal and 1 a reduced one. \(E_1\) and \(E_0\) are supposed to be fully competent in electron transfer, as represented schematically along the four edges limiting the lateral faces \(E_1\) and \(E_2\). The square brackets present in the upper part of the cube indicate a deprotonated state, while the round brackets in the lower part of the cube indicate a protonated state of the enzyme. The vectorial protons are indicated by an arrow and deponents m and e. In the bottom part of the figure, the prevailing pathways populated by the enzyme in the presence of a fully developed electrochemical gradient (S state), or in the presence of valinomycin (P state) are indicated by bold arrows.
experiments (33, 34). To complete the catalytic cycle 2 e⁻ and 4H⁺ (the latter from the M side) are very rapidly transferred to the peroxo intermediate, since when dioxygen is bound the driving force is greatly enhanced. The scheme indicates with deponents m and c only the 4H⁺ which are taken up on the matrix side and released on the cytosolic side (vectorial protons). It is important to notice that the internal electron transfer step, associated with the reduction of the binuclear center is, according to this model, possible also in the E₂ state, i.e. along the left surface of the cube. However when Δψ = 0, the rate constant for the reaction E₁(1100) → E₀(0011) is small enough (k₅ = 10⁻¹ s⁻¹) not to be competitive with the conformational transition (E₁(1100) → E₀(1100)) leading from the input to the output state along the horizontal coordinate, with k₀ = 10⁻² s⁻¹. These values for the rate constants are a close approximation of those observed with COV (under our experimental conditions) for the reduction of the binuclear site, which we maintain to be the rate-limiting step in turnover (13, 26).

According to Brunori et al. (5 see also Ref. 39), the electrochemical gradient stabilizes the enzyme in one conformational state, called S (= slipping), characterized by a slow turnover rate and failure of the proton pump. We propose that S is identical to E₁ and that in the presence of a gradient (>100 mV) a complete turnover involves the reactions along the four edges of the left surface of the cube (as shown in the bottom left of Fig. 4). The stabilization of E₁ (= S) would follow if the rate constant for the transition E₁(1100) → E₀(1100) is inhibited by Δψ dropping from k₀ = 10⁻² s⁻¹ to < 1 s⁻¹, thereby making the internal electron transfer in E₁ (k₅ = 10⁻¹ s⁻¹) the prevailing pathway. From the biochemical viewpoint, the major consequence of freezing the macromolecule in E₁ is the loss of proton translocation, since the output conformation E₀ is never populated significantly during turnover. On the other hand, in the presence of valinomycin (Δψ = 0) the turnover of the enzyme is associated to an oscillation between the two faces of the cube (see Fig. 4, bottom right). Therefore, the P conformation represents a weighted average of the kinetic properties of the two states E₁ and E₀.

This extended version of a cubic scheme for cytochrome oxidase is consistent with available data and presents several interesting features. The fundamental point is the concept that the electrochemical gradient (and notably Δψ) can be treated as an allosteric effector, which controls the conformational state of the enzyme and thereby its redox kinetics and proton translocation efficiency. The effect of membrane potential on the allosteric equilibrium is realized via a control of the rate constant for the transition E₁(1100) → E₀(1100) and thus effectively a change in the relative population of states with a stabilization of E₁. This model predicts that ligand binding to the binuclear center may be under the control of the electrochemical gradient if E₁ and E₀ were equated to the closed and open conformations defined on the basis of the reactivity with cyanide (42, 43). Very recently it was reported by Ray et al. (44) that energization of submitochondrial particles populates (partially) a low-spin state of reduced cytochrome a₅ (possibly affecting accessibility of the binuclear site to oxygen). This important observation is a first direct evidence for a major conformational change of cytochrome oxidase coupled to the electrochemical gradient predicted by Brunori et al. (5). The logical prediction would be that E₁ is characterized by a low-spin reduced cytochrome a₅. Without commenting on the hypothesis that this change in coordination is indeed controlling the rate of respiration, the model suggests that the onset of inhibition by cyanide and the reaction with CO in COV may also be affected by the electrochemical gradient.

Finally, it may be noted that consistency of different models and data sets within one more general scheme is of great value for our understanding of cytochrome oxidase and helps focus the regulatory role of the membrane potential on the activity of membrane-bound enzymes, a phenomenon previously overlooked and possibly of very general significance in biological systems.

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