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Control of Electron Transfer by the Electrochemical Potential Gradient in Cytochrome-*c* Oxidase Reconstituted into Phospholipid Vesicles*

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The kinetics of electron transfer between cytochrome-*c* oxidase and ruthenium hexamine has been characterized using the native enzyme or its cyanide complex either solubilized by detergent (soluble cytochrome oxidase) or reconstituted into artificial phospholipid vesicles (cytochrome oxidase-containing vesicles). $Ru(NH_3)_6^{2+}$ (Ru(II)) reduces oxidized cytochrome *a*, following (by-and-large) bimolecular kinetics; the second order rate constant using the cyanide complex of the enzyme is 1.5×10^6 M^{-1} s^{-1}, for the enzyme in detergent, and slightly higher for COV. In the case of COV the kinetics are not affected by the addition of ionophores.

Upon mixing fully reduced cytochrome oxidase with oxygen (in the presence of excess reductants), the oxidation leading to the pulsed enzyme is followed by a steady state phase and (eventually) by complete re-reduction. When the concentrations of dioxygen and oxidase are sufficiently low (micromolar range), the time course of oxidation can be resolved by stopped flow at room temperature, yielding an apparent bimolecular rate constant of 5×10^7 M^{-1} s^{-1}. After exhaustion of oxygen and end of steady state, re-reduction of the pulsed enzyme by the excess Ru(II) is observed; the concentration dependence shows that the rate of re-reduction is limited at 3 s^{-1} in detergent; this limiting value is assigned to the intramolecular electron transfer process from cytochrome *a*-Cu*a* to the binuclear center. Using the reconstituted enzyme, the internal electron transfer step is sensitive to ionophores, increasing from 2–3 to 7–8 s^{-1} upon addition of valinomycin and carbonyl cyanide *m*-chlorophenylhydrazone. This finding indicates for the first time an effect of the electrochemical potential across the membrane on the internal electron transfer rate; the results are compared with expectations based on the hypothesis formulated by Brunori *et al.* (Brunori, M., Sarti, P., Colosimo, A., Antonini, G., Malatesta, F., Jones, M. G., and Wilson, M. T. (1985) *EMBO J.* 4, 2365–2368), and their bioenergetic relevance is discussed with reference to the proton pumping activity of the enzyme.

Cytochrome-*c* oxidase (EC 1.9.3.1.), the final electron acceptor of the mitochondrial respiratory chain, transfers electrons from cytochrome *c* to molecular oxygen to form water (1, 2). The membrane integrated enzyme pumps protons from the internal (matrix or artificial vesicle lumen) to the external compartment (3–5), synchronously with the redox reaction (6). Therefore, in mitochondria the enzyme contributes to the electrochemical potential gradient which drives ATP synthesis catalyzed by the F_0F_1-ATPase. In spite of extensive investigations (7–9), basic mechanistic questions concerning the electron pathway(s) and the coupling to vectorial translocation of protons are still largely unanswered. It is well known that the activity of the protein, either *in situ* or after reconstitution into phospholipid vesicles, is controlled by the electrochemical potential gradient ($\Delta\tilde{\mu}_{H^+}$) built up during catalysis (10, 11). In its absence the redox activity is maximal, whereas it drops in its presence. The ratio between the rates of cytochrome c^{2+} oxidation or oxygen consumption measured under the two conditions is called "respiratory control ratio" (RCR)[1] (10, 12). In the reconstituted system the RCR reflects, among others, the correct reinsertion of the enzyme in the artificial membrane. As to the mechanism of control, it has been proposed by Brunori *et al.* (13) that cytochrome oxidase activity is regulated by the electrical component of $\Delta\tilde{\mu}_{H^+}$, as a consequence of charge and mass separation between the two membrane surfaces. According to this hypothesis, the reconstituted enzyme may exist in two distinct conformational states, characterized by different catalytic rates and proton pumping efficiency, whose population ratio is modulated by $\Delta\tilde{\mu}_{H^+}$.

To test this hypothesis we have carried out stopped-flow kinetic experiments in order to correlate electron transfer rates with the onset of the electrochemical potential gradient across the membrane and thus to account for the respiratory control ratio. Instead of cytochrome *c*, the spectroscopically silent $Ru(NH_3)_6^{2+}$ has been used as electron donor to reduce cytochrome oxidase (14–16); experiments have been carried out using the enzyme solubilized by the detergent Tween 80 (soluble cytochrome oxidase) or reconstituted into small phospholipid vesicles (COV). Therefore, we have reinvestigated the kinetics of the reaction between Ru(II) and cytochrome oxidase using the cyanide inhibited enzyme, but also in the presence of ascorbate and oxygen as electron donor and acceptor. Analysis of the data obtained with cytochrome oxidase vesicles leads to conclusions about the redox process

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[1] The abbreviations used are: RCR, respiratory control ratio; COV, cytochrome oxidase-containing vesicles; $[Ru(NH_3)_6]^{2+}$ = Ru(II); CCCP = carbonyl cyanide *m*-chlorophenylhydrazone; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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controlled by the electrochemical gradient and, thus, presumably to the reaction step coupled to proton translocation.

MATERIALS AND METHODS

Cytochrome oxidase from beef heart was prepared according to Yonetani (17) with minor modifications; the final pellet was dissolved using 0.1 M phosphate buffer, pH 7.4, containing 0.5% Tween 80. Its concentration is always expressed in (monomeric) functional units. Cytochrome *c* type VI, valinomycin, and CCCP were from Sigma. All other chemicals were of analytical grade.

Ru(II) was prepared by anaerobic reduction of Ru(III), used as purchased by Aldrich, with 5–10 mM ascorbate or with a zinc amalgam under nitrogen (14).

Reconstitution of cytochrome oxidase into vesicles was carried out according to the dialysis method (10) as modified by Casey *et al.* (5). The RCR, measured either spectroscopically or polarographically, was 5 to 6 for different COV preparations. Orientation of cytochrome oxidase (sidedness), indicated on average 85 (±5)% of the molecules to expose cytochrome *a* to the bulk phase (12).

Cyanide-inhibited cytochrome oxidase vesicles: 100 μM protein, solubilized with 0.1 M phosphate buffer, pH 7.4, containing 0.5% Tween 80, was incubated overnight with 15 mM NaCN and then added to the vesicle suspension, just before the dialysis steps. Dialysis buffer contained 50 μM NaCN throughout.

Rapid mixing experiments: all experiments were carried out using a Gibson Durrum stopped-flow apparatus equipped with a 2-cm light-path chamber, thermostatted at 20 °C. The concentration of the heme *a* (2/functional unit) was determined, at 444 nm, using a $\Delta\epsilon$ = 72 mM^{-1} cm^{-1} and 40 mM^{-1} cm^{-1}, respectively, for ($a^{2+}a_3^{2+}$ minus $a^{3+}a_3^{3+}$) and ($a^{2+}a_3^{3+}\cdot CN$) minus ($a^{3+}a_3^{3+}\cdot CN$).

Two protocols for the stopped-flow experiments were followed using both soluble cytochrome oxidase in Tween 80 (soluble cytochrome oxidase) and cytochrome oxidase reconstituted into vesicles (COV): (*a*) the reduction of the oxidized protein, either resting or complexed with cyanide, was followed by mixing the enzyme with Ru(II). (*b*) The protein, fully reduced with Ru(II) and ascorbate, was mixed with buffer containing variable amounts of oxygen and the same reductants (always in excess over oxygen). Under these conditions the enzyme is rapidly oxidized by oxygen and subsequently re-reduced by the excess reductants, the number of turnovers elapsed depending on oxygen and oxidase concentrations. Where necessary ionophores, valinomycin and CCCP, were added to the COV-containing syringe. A typical experiment was carried out as follows: the protein (0.9–1.25 μM), degassed and nitrogen-equilibrated, was reduced in a gas-proof syringe with Ru(II), at different concentrations, in the presence of excess ascorbate (1 mM). The fully reduced protein was incubated for 1 h at room temperature before starting the experiment, in order to achieve complete reduction and dissipation of the membrane potential (eventually developed in COV). The fully reduced protein was then mixed with nitrogen equilibrated buffer containing oxygen, which was progressively and slowly consumed down to zero by catalytic amounts (0.5 nM) of ascorbate oxidase in the presence of ascorbate (5 mM). Unless otherwise specified, all concentrations in the text and figures are after mixing, and all experiments carried out in 10 mM K/Hepes buffer, pH 7.3, made isotonic with the vesicle interior by the addition of 39.6 mM KCl and 40.9 mM sucrose.

RESULTS

Reductive Experiments

(a) Soluble Cytochrome Oxidase—The kinetics of reduction of cytochrome oxidase by Ru(II) has been studied in detail for the enzyme complexed with cyanide, in which the binuclear center is not reduced under the conditions employed (1). The experiments have been carried out by mixing the cyanide-inhibited cytochrome oxidase in air with variable amounts of nitrogen equilibrated Ru(II), in the presence of excess ascorbate. The time courses of the reduction of cytochrome *a* are shown in Fig. 1. When Ru(II) is substoichiometric compared with cytochrome oxidase (*i.e.* from 0.005 to 0.5 μM) (*panel A*), the reaction is monoexponential, and reduction is driven to completion by the excess ascorbate. At higher Ru(II) concentration, the time course tends to deviate from a simple

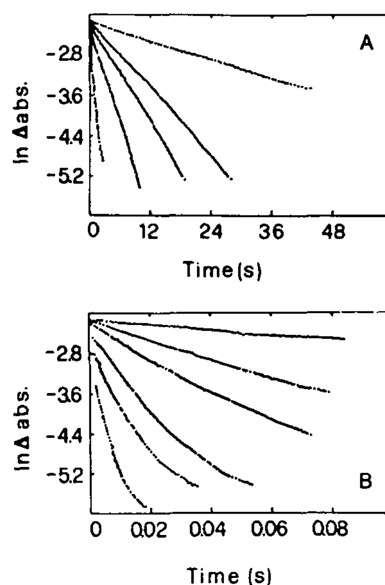


FIG. 1. **Kinetics of reduction of cyanide-inhibited soluble cytochrome oxidase in detergent.** Cytochrome oxidase = 0.75 μM; sodium/ascorbate = 3 mM; Ru(II) micromolar concentrations from *top to bottom* 0.005, 0.05, 0.1, 0.5, and 1.25 (*panel A*) and 5, 10, 20, 50, 100, and 200 (*panel B*). Buffers: 10 mM K^+/HEPES, pH 7.3, containing 39.6 mM KCl, 40.9 mM sucrose and 0.5% Tween 80. Observation wavelength = 444 nm. *T* = 20 °C (see "Materials and Methods" for details).

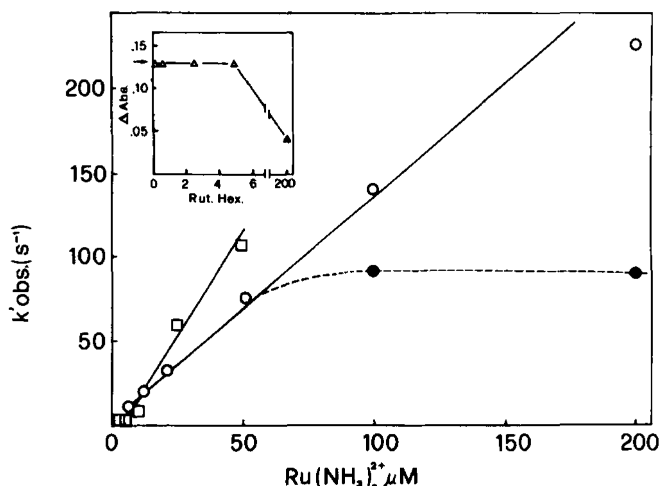


FIG. 2. **Dependence on Ru(II) concentrations of the observed pseudo-first order rate constant for reduction of cyanide-inhibited cytochrome oxidase.** Rate constants are measured for the detergent-solubilized enzyme (*open circles*) and for COV (*open squares*). *Filled circles* refer to the slow phase observed using the soluble enzyme at high concentrations of Ru(II) (see Fig. 1, *panel B*). *Inset* shows the total absorbance change observed at 444 nm as a function of Ru(II) concentration at constant sodium/ascorbate concentration (Δ). *Arrow* indicates the static total absorbance recovery for the same reaction. Experimental conditions are the same as in Figs. 1 and 3.

behavior (*panel B*), especially so at the highest concentrations. The fast kinetic component displays a Ru(II) concentration dependence which is linear up to approximately 100–150 μM (Fig. 2); the second order rate constant for the reduction of cytochrome *a* is the cyanide inhibited enzyme is k = 1.5×10^6 M^{-1} s^{-1}. At the highest concentration of Ru(II) a second kinetic component (which accounts for 25–30% of the reaction and approaches a limiting value of 100 s^{-1}) is well resolved (see Fig. 2).

The observed optical density change at 444 nm, calculated from the stopped-flow experiment, is in very good agreement with the value obtained statically and corresponds to the absorbance change expected for complete reduction of cytochrome *a* (*i.e.* 50% of the total heme *a* content) (1); only at Ru(II) = 200 μM the absorbance recovery decreases to about 40% of the expected value (Fig. 1, *panel B* and *inset* to Fig. 2), since the reaction is partly lost in the dead time of the apparatus.

(b) COV—The reduction of cytochrome *a* was followed by mixing either the oxidized native protein or its cyanide-inhibited derivative (both reconstituted into phospholipid vesicles), with Ru(II) and ascorbate.

When the native oxidized enzyme is mixed with 50 μM Ru(II) and 6 mM ascorbate at low oxygen concentration, the total absorbance change observed at 444 nm corresponds to approximately 60% reduction of cytochrome *a*, and full oxidation of cytochrome a_3, the system approaching a steady state (data not shown). The time course of reduction is insensitive to addition of ionophores (valinomycin and CCCP). When cyanide-inhibited COV are mixed with Ru(II) and ascorbate in excess over oxidase, the observed process (Fig. 3) corresponds within the errors to a first order reaction with complete recovery of the expected absorbance change at 444 nm. In order to confirm that the latter process is contributed only by the reduction of cytochrome *a*, the kinetic difference spectrum has been compared with the static one ($a^{2+}a_3^{3+}\cdot$CN minus $a^{3+}a^{3+}\cdot$CN) obtained with the same preparation of COV. The *inset* to Fig. 3 shows that the difference spectrum in the presence and absence of ionophores is identical and very similar to the reduced-minus-oxidized difference spectrum of cytochrome *a* as reported in literature (1).

The effect of Ru(II) concentration on the rate constant for reduction of cytochrome *a* in cyanide-inhibited COV has also been investigated. Fig. 2 also shows the pseudo-first order rate constant for reduction of COV, as a function of Ru(II) concentration, at constant ascorbate (3 mM). The data, obtained at 605 and 444 nm, display a complex concentration dependence. In the lower concentration range (up to approximately 5 μM Ru(II) the observed pseudo-first order rate constant is very low and insensitive to Ru(II) concentration, whereas at higher concentrations it increases linearly (as expected). The very small rate of reduction of cytochrome *a* obtained at low Ru(II) concentrations is compatible with the rate of reduction by sodium/ascorbate alone, as determined independently. This complex concentration dependence is rationalized by the finding that the (negatively charged) vesicles bind Ru(II) on their surface. This was shown by the fact that addition of phospholipid vesicles not containing cytochrome oxidase to the Ru(II) containing syringe reduces the rate of reduction of the enzyme, presumably by "sequestering" a fraction of the reductant. Once the binding capacity of the vesicles is saturated, further increase of Ru(II) concentration leads to an increase of the observed rate constant for cytochrome *a* reductions as shown in Fig. 2. The reduction follows bimolecular behavior, and the second order rate constant is $k = 2.5 \times 10^6$ M^{-1} s^{-1}; thus, quite similar to the value measured in solution. The rate of reduction of cytochrome *a* by Ru(II) is independent of ionophores, over the whole reductant concentration range.

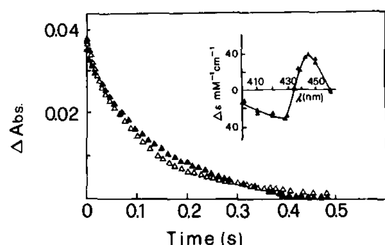


FIG. 3. **Time courses of reduction of cyanide-inhibited cytochrome oxidase in vesicles.** The reaction is followed at 444 nm in the absence (▲) and presence (△) of 5 μM valinomycin and 5 μM CCCP. Cytochrome oxidase = 0.5 μM; Ru(II) = 10 μM; sodium/ascorbate = 3 mM. *Inset* shows the kinetic difference spectrum for the reduction process observed in the presence and absence of ionophores. The static difference spectrum (*continuous line*) is reported for comparison; T = 20 °C (details under the "Materials and Methods").

Turnover Experiments

(a) Soluble Cytochrome Oxidase—Following the protocol detailed under the "Materials and Methods," the oxidation of fully reduced cytochrome oxidase was monitored by stopped-flow, by lowering progressively the oxygen concentration with ascorbate oxidase. Fig. 4 (*panel A*) shows the time courses of the oxidation phase followed at 444 nm at different oxygen concentrations. When oxygen is in large excess, most of the reaction is lost in the dead time of the apparatus (traces above the *arrow* in Fig. 4, *panel A*). When oxygen is approximately stoichiometric with the oxidase (trace labeled with an *arrow* in Fig. 4, *panel A*), and both are in the micromolar concentration range, oxidation occurs with a $t_{1/2}$ of about 10 ms, and Δabsorbance recovery is maximal. Treatment of the oxidation reaction as a second order irreversible process, yields a bi-

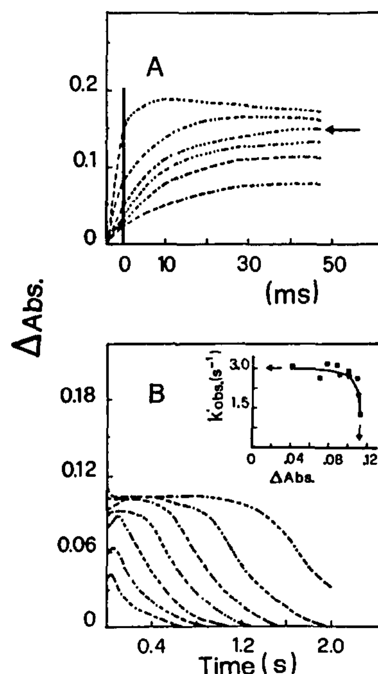


FIG. 4. **Oxidation (*panel A*), steady state, and re-reduction (*panel B*) time courses of soluble cytochrome oxidase in detergent, at different oxygen concentrations.** Cytochrome oxidase = 0.6 μM; Ru(II) = 5 μM (*panel A*) and 100 μM (*panel B*). Sodium/ascorbate = 3 mM. Ascorbate oxidase = 0.25 nM. In *panel A*, *zero* on the time axis indicates when observation starts (*solid line*); the dead time of the apparatus is 4 ms. The *arrow* indicates the trace showing the maximal Δabsorbance recovery corresponding to a dioxygen/protein stoichiometry close to 1:1 (see text). In *panel B*, the *inset* shows the dependence of the observed re-reduction rate constant on the total absorbance change recovery (see text). In both panels oxygen concentration decreased from *top* to *bottom* and ranges from substoichiometric relative to the protein (*i.e.* lower than 0.6 μM) up to several molar excesses (details under "Materials and Methods"). Observation at 444 nm, T = 20 °C.

molecular rate constant of 5.5×10^7 M^{-1} s^{-1}. On a longer time scale (see Fig. 4, *panel B*) a steady state phase is detected when oxygen exceeds the concentration of the enzyme; once oxygen is exhausted, the enzyme is completely re-reduced, as a result of the large excess of reductants. The duration of the steady state and the time course of re-reduction are correlated to the initial oxygen concentration. Of course, when oxygen is less than stoichiometric, only a fraction of the total enzyme is oxidized and subsequently re-reduced and Δabsorbance recovery therefore tends to zero (see *bottom traces* in Fig. 4, *panels A* and *B*).

The re-reduction rate has been calculated from the slope of the semilogarithmic plots of traces in *panel B*. It appears that the re-reduction rate constant increases as the oxygen concentration (and therefore length of the steady state) decreases, reaching a constant value when oxygen is substoichiometric relative to oxidase (*inset* to Fig. 4*B*). Fig. 5 shows the dependence of Ru(II) concentration of the first order rate constant for the re-reduction phase measured under the latter conditions. The rate of re-reduction increases with the concentration of Ru(II) below 25 μM and thereafter is concentration-independent ($k = 3$ s^{-1}).

(b) COV—Following the same protocol, the experiments were carried out by mixing an anaerobic suspension of COV in the presence of 10 μM Ru(II) and 1 mM sodium/ascorbate, with a buffer containing oxygen, 5 mM Na/ascorbate and 0.5 nM Ascorbate oxidase (see "Materials and Methods"). Fig. 6 shows the oxidation phase observed in the presence and absence of ionophores, when oxygen concentration is close to stoichiometric with the oxidase. Given the low concentrations of reactants, the reaction is followed for approximately 75% of its full extent and proves to be clearly insensitive to ionophores. The second order rate constant calculated for the oxidation is 4.8×10^7 M^{-1} s^{-1} (±1.0), average of four determinations, a value close to the one measured for the enzyme in detergent (see above).

Similar to what was observed in solution, once oxygen is exhausted COV become re-reduced in the presence of excess reductants. The time course of this process (not shown) is almost completely superimposable in shape to those described above for the enzyme in detergent. Fig. 6 also shows the time

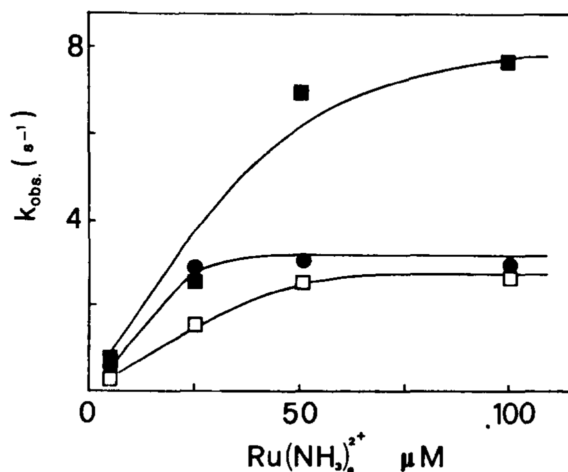


FIG. 5. **Dependence on Ru(II) concentration of the pseudo-first order rate constant for re-reduction of cytochrome oxidase.** Rate constants are measured for the detergent-solubilized enzyme (0.6 μM) (*circles*) and for COV (0.45 μM) (*squares*). COV experiments were carried out in the absence (□) and presence (■) of 5 μM valinomycin and 5 μM CCCP. Values of k'_{obs} were obtained from plots of the type shown in the *inset* to Fig. 4 (*panel B*) (details under "Materials and Methods").

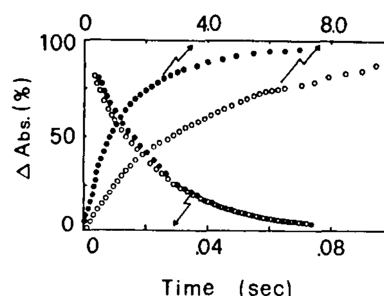


FIG. 6. **Time courses of oxidation (lower time scale) and re-reduction (upper time scale) of cytochrome oxidase in vesicles, in the presence (●) and absence (○) of ionophores.** Cytochrome oxidase = 0.45 μM; Ru(II) = 5 μM; sodium/ascorbate 3 mM; Ascorbate oxidase = 0.25 nM. Ionophores concentration as in Fig. 5.

TABLE I

Summary of the measured rate constants

k	SOX[a]	COV	COV + ionophores
Reduction of cytochrome a^b (M^{-1} s^{-1})	1.5×10^6	2.5×10^6	2.5×10^6
Oxidation of fully reduced oxidase (M^{-1} s^{-1})	5.5×10^7	4.8×10^7	4.8×10^7
Reduction of cytochrome a_3 (s^{-1})	3	2–3	7–8

[a] SOX, soluble cytochrome oxidase.
[b] Cyanide-inhibited enzyme.

courses of re-reduction in the presence and absence of ionophores, as observed at 444 nm and at the same oxygen concentration (substoichiometric with the enzyme). The semilogarithmic treatment of the progress curve shows that the reaction can be closely described by one exponential process, although at the highest Ru(II) concentration a second kinetic component (slower by a factor of 2 and accounting for as much as 20–30% of the absorbance change) is detected. The pseudo-first order rate constant for re-reduction obtained at different Ru(II) concentrations, shown in Fig. 5, indicates saturation kinetics, the limiting values being at 2–3 s^{-1} and 7–8 s^{-1} in the absence and presence of ionophores, respectively. Table I summarizes the most relevant rate constant values measured in this study.

DISCUSSION

Kinetic Characterization—The kinetics of reduction by Ru(II) has been more extensively investigated when cyanide is bound to the binuclear center, keeping cytochrome a_3 in the ferric state. Under these conditions, mixing Ru(II) (in the presence or absence of excess ascorbate) with the cyanide complex of the detergent solubilized enzyme leads to complete reduction of cytochrome *a* and thus presumably also of Cu_A. The kinetics conform to a psuedo-first order model over a large range of Ru(II) concentrations. In agreement with previous data (14, 16) the pseudo-first order rate constant for the reduction of cytochrome *a* depends linearly on the concentration of Ru(II), yielding a second order rate constant of 1.5×10^6 M^{-1} s^{-1} (see Fig. 2). A second kinetic component is clearly observed in the reduction process only at high Ru(II) concentrations (>50 μM), with an observed rate constant of approximately 100 s^{-1}. At present we have no simple explanation for this behavior, apart from possible kinetic heterogeneity manifested only at high reductant concentration.

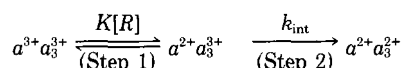
The reduction of the cyanide-inhibited COV by Ru(II) is more complex, because binding to the vesicles (approximately 5 μM ruthenium hexamine with 6 mg/ml of asolectin) seques-

ters some of the reductant from reaction with the oxidase. The less favorable optical properties of the vesicle suspension make observations more difficult when the rate constant is above 100 s^{-1}. However, the second order rate constant calculated from the data in Fig. 4 ($k = 2.5 \times 10^6$ M^{-1} s^{-1}) is similar to that determined for the soluble enzyme.

In the oxidation experiments, the oxygen concentration was changed progressively from excess to substoichiometric; at approximately stoichiometric oxygen, the oxidation of cytochrome oxidase in solution and in COV occurs in the millisecond time range. In all these experiments the initial product of oxidation is the *pulsed* enzyme (18, 19). Absorbance recovery is a function of oxygen concentration, being minimal at high oxygen since most of the reaction is lost in the dead time of the apparatus (see Fig. 4); upon decrease in oxygen concentration, the observed absorbance change goes through a maximum, and then decreases (eventually down to zero) when oxygen is substoichiometric. On the assumption of irreversible bimolecular kinetics, the rate constant calculated for the oxidation of soluble and reconstituted cytochrome oxidase when dioxygen is stoichiometric with the protein ranges from 4.8 to 5.5×10^7 M^{-1} s^{-1}; compared to the value originally reported by Gibson and Greenwood (20) for the oxidation of cytochrome a_3, this range is only slightly smaller (2-fold). In spite of some complexities and new features emerged from subsequent studies (21–23) of the oxidation reaction followed in flow-flash experiments, it seems likely that under our conditions the process of combination of oxygen with reduced cytochrome a_3 is rate-limiting, given the very low dioxygen concentrations employed. This interpretation is supported by the recovery of the optical density change at 444 nm and by the satisfactory argreement of the calculated second order rate constant with the value obtained by flow-flash starting from the CO derivative (20–23).

The time course of the steady-state phase (when present) and that of the re-reduction of the protein are complex, given that we are dealing with competing kinetic events. Assuming that cytochrome a_3 is fully oxidized during turnover, absorbance recovery indicates that at steady-state the fraction of oxidized cytochrome a ranges from 80 to 40% going from low to high Ru(II) concentrations

The time course of re-reduction, as observed at substoichiometric oxygen in solution and COV, has been analyzed as a single exponential phase. The absence of a biphasic time course (even at the highest Ru(II) concentrations) suggests that the reduction of cytochrome a competes successfully with its oxidation, since at low oxygen concentrations oxidation of cytochrome a is rate-limited by the binding of dioxygen to reduced cytochrome a_3 (see above). The first order rate constant for re-reduction increases at low Ru(II) concentration and thereafter tends to a plateau. The limiting value observed for COV in the presence of ionophores ($k = 7.5$ s^{-1}) is higher than that for the enzyme in detergent ($k = 2.5$ s^{-1}); we assign this difference to phospholipid activation of the electron transfer rate in cytochrome oxidase, in agreement with previous data (12). The overall kinetic behavior of re-reduction may be described by the following scheme



where $[R]$ = Ru(II) concentration, K = Ru(II) association equilibrium constant, k_{int} = rate constant for reduction of cytochrome a_3. Such a scheme may be justified by the fact that the final re-reduction process follows first order behavior at all Ru(II) concentrations and that the reduction of cytochrome a, as determined independently, is always much faster than reduction of cytochrome a_3 (Figs. 2 and 5); thus, step 1 in the above scheme is always close to equilibrium relative to step 2. Within this approximation it may be shown that the apparent rate constant for re-reduction is given by

$$k_{obs} = k_{int} \cdot \frac{K[R]}{K[R] + 1}$$

accounting for the initial concentration dependence of the internal electron transfer process. When $[R]$ approaches to zero the initial slopes of the plot in Fig. 5 will approach $dk_{obs}/d[R] = k_{int} \times K$. Since an estimate of k_{int} is obtained at high Ru(II) concentrations, the equilibrium constant K for binding and electron transfer from Ru(II) to cytochrome a^{3+} can be calculated. This calculation yields $K = 2$ and 3×10^4 M^{-1} in the absence and presence at ionophores, respectively; this finding indicates that the development of a membrane potential by electron transfer does not dramatically affect K but rather regulates the magnitude of the internal electron transfer rate constant (k_{int}).

In summary, the observed re-reduction is assigned to the internal electron transfer (k_{int}) from cytochrome a (and Cua) to internal electron transfer (k_{int}) from cytochrome a (and Cua) to cytochrome a_3 (and Cua_3) in the *pulsed* enzyme on the basis of the following considerations: (a) after the oxygen pulse this process occurs with a rate constant (from 2 to 8 s^{-1}) compatible with previous estimates for the reduction of cytochrome a_3 (18, 24, 25); (b) under our conditions, reduction of cytochrome a by Ru(II) is much faster ($\gg 10$ s^{-1}); (c) re-reduction is clearly detected under conditions in which cytochrome a is significantly or largely reduced at steady-state; (d) its amplitude is consistent with the spectral contribution of cytochrome a_3 at 444 nm; (e) the observed rate constant is consistent with the turnover number of the enzyme under similar conditions (25); and (f) the observed rate constant becomes independent of oxygen concentration when this is substoichiometric

Bioenergetic Relevance—The experiments carried out with cytochrome oxidase reconstituted into vesicles provide an opportunity to probe the effect of the membrane potential on the redox kinetics of the enzyme. In spite of complexities due to the binding of Ru(II) to the vesicles (see Fig. 2), the kinetics of reduction of the CN-inhibited enzyme in COV yields results consistent with a bimolecular process involving the reduction of cytochrome a (and Cua) by Ru(II) with a second order rate constant of 2.5×10^6 M^{-1} s^{-1}. This process is not affected by ionophores, as shown by the identity of the time courses observed in the presence and absence of valinomycin and CCCP at micromolar concentrations (see Fig. 3). This observation is not surprising, because the location of cytochrome a and Cua is supposed to be close to the external surface of the membrane (26, 27); this topology is not expected to be associated to transfer of electrons across the bilayer during the reduction of these two metal centers and thus to generate $\Delta\tilde{\mu}_{H^+}$. Likewise the oxidation of the reduced COV by oxygen (discussed extensively above) is insensitive to ionophores (see Fig. 6). Given the fact that under our conditions the bimolecular oxidation process is rate-limiting, no information is afforded by the data on a possible effect of the gradient on the rate of oxidation of cytochrome a (which proceeds at room temperature at a rate ≥ 700 s^{-1}) (20, 22, 23).

As discussed above, the re-reduction at substoichiometric oxygen is assigned to the internal electron transfer from reduced cytochrome a-Cua to the binuclear centre, whose rate constant tends to a limiting value at high Ru(II) concentrations. In the case of COV, the internal electron transfer rate constant (k_{int}) is clearly different in the absence and presence

of ionophores (Fig. 6), indicating that this step is indeed controlled by the gradient produced during the oxidation phase leading to the *pulsed* enzyme. Thus, the rate of reduction of cytochrome a_3 is increased by the addition of ionophores by a factor of 2–3 (*i.e.* from 2–3 s^{-1} to 7–8 s^{-1}). It is tempting to correlate this observation with the respiratory control of COV and to analyze its consistency with the expectations based on the model proposed by Brunori *et al.* (13) for the linkage between membrane potential and functional properties of cytochrome oxidase.

The RCR of the preparations of COV used in these experiments (as determined following either the oxidation of ferrocytochrome c^{2+} or the oxygen consumption) is 5–6. According to the model (13), the respiratory control in COV is solely accounted for by an effect of $\Delta\tilde{\mu}_{H^+}$ on the rate of the internal electron transfer from cytochrome *a* (and/or Cu*a*) to cytochrome a_3-Cua_3 (k_{int}). The build-up of the gradient during turnover leads to the stabilization of a conformational state of cytochrome oxidase (called S), which is characterized by a depressed value of k_{int} as compared with the gradient-free state (called P). Since only upon addition of valinomycin cytochrome oxidase was shown to translocate protons, both in mitochondria and in COV (3–6), it was proposed (13) that the enzyme in the S (slipping) state is not a proton pump contrary to the P (pumping) state, which is fully populated in the presence of ionophores; thus, in the S state a slipping mechanism (28) is operative. Interesting enough, recent data by Murphy and Brand (29) using rat liver mitochondria have been interpreted to indicate that the H^+/e^- ratio in cytochrome oxidase is a function of the steady state membrane potential, and proton translocation by Complex IV is suppressed at high membrane potential (*i.e.* 180 mV under their experimental conditions).

The transient kinetic data reported above appear to us consistent with the premises of our working hypothesis (13), in so far as the internal electron transfer from cytochrome *a*-Cu*a* to cytochrome a_3-Cua_3, is the only kinetic event which is seen to be controlled by the transmembrane electrochemical gradient (as shown in Fig. 6). We now examine some quantitative aspects of this observation. Addition of ionophores increases the value of k_{int} by a factor of 2–3 (see Fig. 5); this increase in the long-range electron transfer rate is about one-half the value of the RCR (5–6 for our COV preparation). It is widely accepted that oxidation of 1 functional unit of the enzyme leads to translocation of 4 electrons across the membrane. Given that cytochrome oxidase is reconstituted (at least) as a dimer (corresponding to 2 functional units) (30, 31), and assuming an ideally homogeneous population of COV containing one dimer/vesicle (but see Ref. 32), the minimum membrane potential generated in the oxidation of one dimer/vesicle would correspond to translocation of 8 electrons, *i.e.* approximately 80 mV given the average physical properties of the vesicles (see Ref. 32). If proton translocation (with a $H^+/e^- = 1$) occurred in synchrony with oxidation by oxygen, the membrane potential generated during oxidation of reduced cytochrome oxidase would be twice as much. According to our model, based on the interpretation of stopped-flow experiments with COV, at 160 mV only the S state should be populated (13, 34); thus, in the absence of ionophores k_{int} should be lower than the maximal value achieved in the presence of ionophores (k_{int} = 7–8 s^{-1}) by a factor corresponding to the respiratory control ratio. The observation that the effect of ionophores on k_{int} is smaller may be interpreted to indicate that, on average, the P and S states are both populated after the oxidation phase; indeed our model predicts that P and S would be equally populated at a membrane potential = 80 mV (34).

A rationale for this quantitative inconsistency may be offered *if proton translocation were not coupled to oxidation of cytochrome oxidase* and therefore if proton pumping occurred only during the reduction of (some of) the metal centers in the translocation cycle. Albeit speculative, this novel possibility leads to predictions which may be verified by experiment. Thus, no proton pumping should be observed during oxidation of COV in flow-flash experiments, which is accessible to transient kinetic studies. Moveover, experiments similar to those reported above, but carried out with COV characterized by very different values of the RCR (say from 4 to 14), should lead to quantitatively different effects of the ionophores on k_{int}. Finally, k_{int} should be a predictable function of the membrane potential (35), even when imposed by some external means (such as co-reconstitution with bacteriorhodopsin, see Ref. 36).

In conclusion, the experiments with COV have clearly shown that the internal electron transfer step in cytochrome oxidase is under the control of the electrochemical potential across the membrane, in agreement with the hypothesis of Brunori *et al.* (13, 34). Moreover, quantitative considerations of these kinetic results, and comparison with the measured value of the RCR, lead to the prediction that the oxidation phase of the turnover cycle would not be associated to translocation of protons into the bulk, and thus, this important function would be coupled to kinetic event(s) involved in the reduction of some of the metal center(s) of the enzyme.

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