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Flash-induced membrane potential generation by cytochrome *c* oxidase

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Flash-induced single-electron reduction of cytochrome *c* oxidase. Compound **F** (oxoferryl state) by $Ru^{II}(2,2'\text{-bipyridyl})_3^{2+}$ [Nilsson (1992) Proc. Natl. Acad. Sci. USA 89, 6497–6501] gives rise to three phases of membrane potential generation in proteoliposomes with τ values and contributions of ca. 45 μs (20%), 1 ms (20%) and 5 ms (60%). The rapid phase is not sensitive to the binuclear centre ligands, such as cyanide or peroxide, and is assigned to vectorial electron transfer from Cu_A to heme *a*. The two slow phases kinetically match reoxidation of heme *a*, require added H_2O_2 or methyl peroxide for full development, and are completely inhibited by cyanide; evidently, they are associated with the reduction of Compound **F** to the **Ox** state by heme *a*. The charge transfer steps associated with the **F** to **Ox** conversion are likely to comprise (i) electrogenic uptake of a 'chemical' proton from the *N* phase required for protonation of the reduced oxygen atom and (ii) electrogenic H^+ pumping across the membrane linked to the **F** to **Ox** transition. Assuming heme *a* 'electrical location' in the middle of the dielectric barrier, the ratio of the rapid to slow electrogenic phase amplitudes indicates that the **F** to **Ox** transition is linked to transmembrane translocation of 1.5 charges (protons) in addition to an electrogenic uptake of one 'chemical' proton required to form $Fe^{3+}\text{-}OH^-$ from $Fe^{4+}{=}O^{2-}$. The shortfall in the number of pumped protons and the biphasic kinetics of the millisecond part of the electric response matching biphasic reoxidation of heme *a* may indicate the presence of 2 forms of Compound **F**, reduction of only one of which being linked to full proton pumping.

Cytochrome *c* oxidase; Membrane potential; Oxygen intermediates; Proton pumping; Ruthenium photochemistry; Rapid kinetics

1. INTRODUCTION

Cytochrome *c* oxidase (COX), a terminal oxidase of the respiratory chain of mitochondria and many bacteria, catalyses 4 e^- reduction of O_2 to water coupled to electrogenic pumping of protons across the membrane [1–4].

Since its discovery by Wikstrom in 1977 [5], the electrogenic H^+ translocation by COX has been an issue of singular appeal in bioenergetics and a vast number of hypothetical mechanisms have been advanced. Notably, each of the four metal redox sites of the enzyme (heme *a*, Cu_A, heme a_3 and Cu_B) has been considered as the key element of the proton pumping machine (cf. [1–3,6–12]), but at present the energy-coupling is thought to be associated with the oxygen intermediate interconversions in the heme a_3/Cu_B binuclear centre. According to Wikstrom [13], proton pumping by COX is linked specifically to the **P** → **F** and **F** → **Ox** transitions of the enzyme, each of these single-electron steps being coupled to translocation of 2 H^+ ions across the membrane.

Time-resolved measurements of charge transfer by COX would be most helpful for elucidation of the enzyme electrogenic mechanism. The problem is that the reaction catalysed by COX is too rapid to be followed with the conventional membrane potential probes while the enzyme does not contain endogenous $\Delta\psi$ indicators, like carotenoids in the photosynthetic bacteria reaction centres.

An electrometric method of monitoring electrogenic activity of membrane-bound energy-transducing enzymes has been developed in this laboratory [14,15] and adapted for time-resolved studies of $\Delta\psi$ generation by bacteriorhodopsin [16] and reaction centre [17,18] or cytochrome bc_1 [19] complexes from photosynthetic bacteria.

Previously, the assay was used to demonstrate generation of $\Delta\psi$ by COX in proteoliposomes [20] or submitochondrial particles [21] under steady-state conditions. Here we describe the association of COV with phospholipid-impregnated collodion film and report on the rapid electrogenic processes linked to flash-induced reduction of COX Compound **F** to the **Ox** form by RuBpy.

2. MATERIALS AND METHODS

2.1. *Chemicals, preparations*

$Ru^{II}(2,2'\text{-Bipyridyl})_3Cl_2$ (Aldrich), H_2O_2 (30%, 'Suprapur', Merck) egg yolk lecithin (Reachim), stearyl amine and asolectin (Sigma) were used. Other chemicals were mainly from Sigma, Serva and Fluka. COX was isolated from beef heart mitochondria [22,23] and reconstituted into asolectin liposomes by a cholate dialysis method [24].

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Abbreviations: COX, cytochrome *c* oxidase; COV, cytochrome *c* oxidase vesicles; RuBpy, $Ru^{II}(2,2'\text{-bipyridyl})_3^{2+}$; **P**, **F**, **Ox**; peroxy, oxoferryl and oxidized states of COX; $\Delta\psi$, transmembrane electric potential difference.

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2.2. *Electrometric measurements*

A method and a set up for the electrometric measurement of the membrane potential have been described [14–18]. Briefly, an opening in a partition separating two 4.5 ml compartments of a teflon cell with 2 optical windows is closed with a collodion film soaked in a decane solution of asolectin (100 mg/ml), or egg yolk lecithin (100 mg/ml) + stearyl amine (1 mg/ml). COV are adhered to one side of the film for a 3–5 h incubation at a conc. of ca. 1 μM COX, in buffer containing 10 mM HEPES-KOH and 30 mM Ca^{2+} or Mg^{2+}. After the incubation, excess of the unbound vesicles was removed, and both electrometric cell compartments were refilled with a final reaction buffer using a peristaltic pump.

Electric potential difference between the two compartments is measured with a pair of light protected Ag/AgCl electrodes. The signal is fed into an operational amplifier (Burr-Brown 3554) and then into a Datalab-1080 transient digitizer linked to an IBM PC computer.

2.3. *Flash-activated electron injection into COX*

We have used an approach described recently by T. Nilsson [25] and based on previous work on the Ru(II) polypyridyl compound photochemistry [26–29]. COX or COV were preincubated with RuBpy which binds to the enzyme at the cytochrome *c* docking site on Cu_A. Laser flashes delivered by a YAG laser (Quantel, 481) operated at a doubled frequency (λ = 532 nm; pulse half-width, 15 ns; ca. 50 mJ/flash) excite Ru(II)Bpy to a state with E_m of about −1 V which reduces Cu_A in less than 1 μs [25]; the Ru(III)Bpy formed is re-reduced to the Ru(II)Bpy by a sacrificial donor (10 mM aniline) making electron injection into COX irreversible. Subsequent electron redistribution within COX is monitored electrometrically, or optically in a custom-built spectrophotometer (e.g. see [18]).

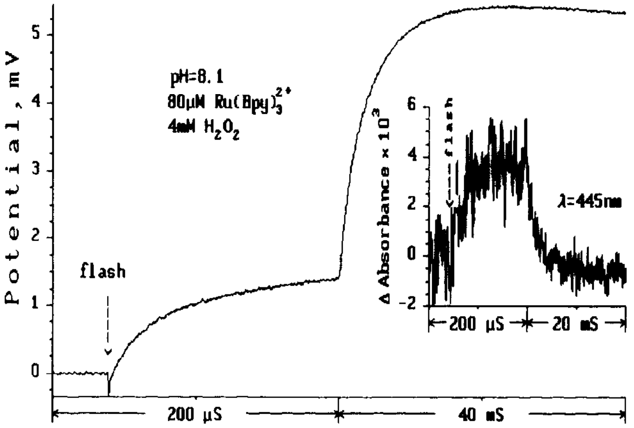

Fig. 1. Flash-induced electrogenic reduction of COX Compound F to the oxidized state. COV have been adhered to one side of the collodion film soaked in a phosphatidyl choline/stearyl amine solution in decane. Both compartments are filled with 5 mM Tris-acetate pH 8. 80 μM RuBpy, 10 mM aniline and 4 mM H_2O_2 have been added ca. 5 min before the flash. The experimental trace (an average of 3 runs) has been fitted to three-exponent kinetics with the parameters found listed in the Table I. Inset: flash-induced transient reduction of cytochrome *a* by RuBpy in Compound F. The 1 cm optical cell contained 5 μM COX in 5 mM Tris-acetate pH 8.1, 0.5% Tween-80 supplemented with 80 μM RuBpy and 10 mM aniline. 4 mM H_2O_2 has been added to form Compound F. The curve is an average of 50 transients.

3. RESULTS

In agreement with Nilsson [25], we found that flashing COX, preequilibrated with 80 μM RuBpy at low ionic strength, brings about transient reduction of heme *a* (τ ca. 50 μs); in Compound F, this is followed by complete reoxidation of the heme with an apparent half-time of 3–5 ms (Fig. 1, inset).

We then attempted to follow electrogenic processes associated with the flash-induced reduction of F in COV. It has been found that after a prolonged incubation, COV adhere to the collodion film impregnated with a decane solution of phospholipids. Addition of ascorbate and cytochrome *c* and/or $Ru(NH_3)_6^{2+}$ results in a steady-state electric potential difference generated across the collodion film (up to 50 mV, positive on the COV side), which is inhibited by KCN (not shown).

Flashing the collodion film-adhered COV preequilibrated with RuBpy and treated by 4 mM H_2O_2 to convert COX to the F state [30,31] gives rise to a rapid electric response corresponding to translocation of positive charges from the inside to the outside of the vesicles (Fig. 1). An initial negative transient (probably, an artifact) is followed by a rapid phase of $\Delta\psi$ generation (τ ca. 45 μs) and a slower part with an apparent $t_{1/2}$ of about 3 ms.

Deconvolution of the trace in Fig. 1 reveals 3 exponents with τ and relative amplitudes (α) values given in Table I. Consistent results have been obtained in about 10 experiments with several COV preparations. Nota-

Table I

Flash-induced electron injection into cytochrome *c* oxidase compound F

	Electric response		Absorption measurements ([25], this work)	
Phase	τ	α (contribution)	τ	Process
Rapid	43 μs	0.20	43 – 47 μs	heme *a* reduction
	44 μs (36 ± 48)	0.21 (0.19 ± 0.25)		
Intermediate	1.2 ms	0.20	0.77 ms	heme *a* reoxidation
	1.1 ms (0.9 ± 1.3)	0.22 (0.19 ± 0.25)		
Slow	5.1 ms	0.60	3 ms	
	4.9 ms (4.3 ± 5.6)	0.57 (0.50 ± 0.60)		

See Fig. 1 and section 2 for conditions. For each phase of the electric response, the upper line gives the values of τ and α found for the curve shown in Fig. 1, whereas the lower lines give the mean values (plus scatter interval in brackets) for 10 experiments with different samples. Note that τ is a time constant, $t_{1/e}$, not a half-time, $t_{1/2}$.

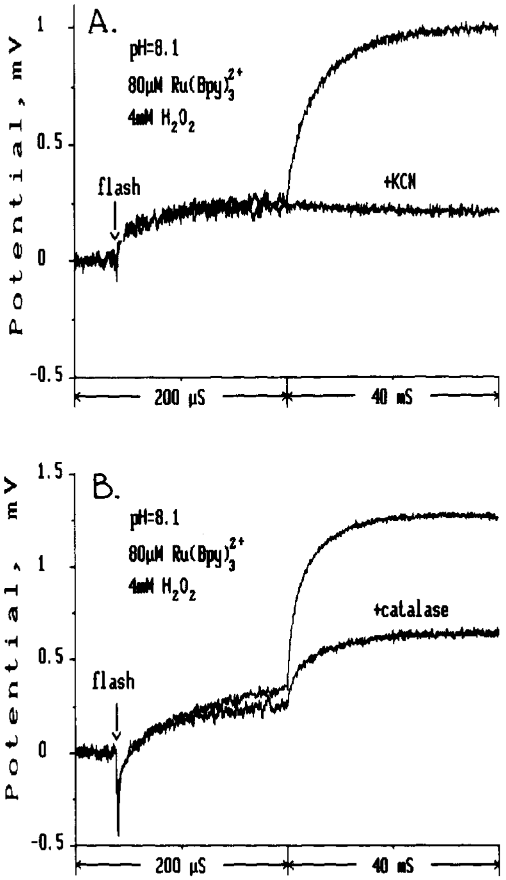

Fig. 2. Effect of cyanide and catalase on the flash-induced electrogenic response of COX. Basic conditions, as in Fig. 1. (A) Where indicated, 200 μM KCN has been added 10 min before the flash; the buffer has been supplemented with 200 μM ferricyanide to prevent spontaneous reduction of heme *a*. (B) Where indicated, catalase has been added after H_2O_2 (5 μl from the 'Sigma C-100' stock suspension freshly diluted 1:50) and 20 min incubation was allowed before the flash. The traces are the average of 3 runs.

bly, there is a good correspondence between the three electrogenic phases and the optically measured phases of reduction and re-oxidation of heme *a* reported in [25] and reproduced by ourselves.

The 45 μs phase is not affected by cyanide, whereas the millisecond part of the electric response is fully inhibited by 200 μM KCN (Fig. 2A). Also the rapid phase is not sensitive to catalase, which induces decay of the **F** state generated by H_2O_2. In contrast, the millisecond part of $\Delta\psi$ generation diminishes several-fold upon addition of catalase (Fig. 2B), although we failed to obtain full inhibition. It is noteworthy, that the kinetics of the residual catalase-resistant slow part of the electric response does not differ significantly from that in the initial trace, i.e. there is no evidence for the 1 and 5 ms components being affected differently. Presumably, part of COX remain in the **F** or **P** state despite the presence of catalase, which is not unfrequently observed with the liposome-bound enzyme (Vygodina, unpublished).

Addition of methyl peroxide after catalase, restoring the **F** state [32], greatly enhances the millisecond electric phase but does not affect either the amplitude or the rate of the 45 μs phase (data not included).

4. DISCUSSION

4.1. *Identification of the electrogenic phases*

Generally, the electric responses associated with the reduction of compound **F** in COV correlate reasonably well with the electron transfer events measured optically in the solubilized COX (Table I). Three major charge transfer steps are revealed with τ values of ca. 45 μs, 1 ms and 5 ms.

We attribute the 45 μs phase to reduction of heme *a* (Fig. 3). First, its rate is close to that of electron transfer from Cu_A to heme *a* ([25,29], this work). Second, this phase is not affected significantly by the binuclear a_3/Cu_B centre ligands, such as cyanide or H_2O_2.

The slow part of the electric response is likely to be associated with reoxidation of the flash-reduced heme *a* by the ferryl heme a_3. In particular it reveals 2 phases with τ values similar to those observed for heme *a* reoxidation in Compound **F** (Table I). Consistent with this interpretation, the slow part of the electric response is largest in the presence of excess H_2O_2 or MeOOH, and is fully inhibited by cyanide.

4.2. *Quantitation of the electric response*

The magnitude of the overall electric response ($\Delta\psi$) induced by single-electron reduction of **F** to **Ox** by RuBpy can be expressed to a first approximation as

$$\Delta\psi \ \alpha \ 1 \times l + n + m \times (1 - l) \qquad (1)$$

where *l* is an 'electrical' distance between heme *a* and the *P*-phase: $l_a = (\psi_a - \psi_P)/\psi_N - \psi_P)$, *n* is the number of the pumped protons and *m* is the number of 'chemical' protons uptaken by **F** upon its reduction to **Ox** (see Fig. 3).

Contribution, α, of the rapid electric phase (heme *a* reduction) is

$$\alpha_{\text{rapid}} = \frac{l}{l + n + m \times (1 - l)} \qquad (2)$$

which allows to put some boundaries on the stoichiometry of 'chemical' and pumped protons in Fig. 3.

According to the structural data, heme *a* is localized rather close to the *P*-phase [4]. However, the 'electrical' distance ratios may differ significantly from the spatial ones due to variation in the intraprotein dielectric constant along the charge transfer traectory, as has been demonstrated for instance for bacteriorhodopsin (cf. [36,37]) or *R. viridis* reaction centres [18]. According to the classical work of Hinkle and Mitchell [33], heme *a* is localized electrically in the middle of the COX dielectric barrier, so we assume $l = 0.5$ in Eq. (2). Taking a

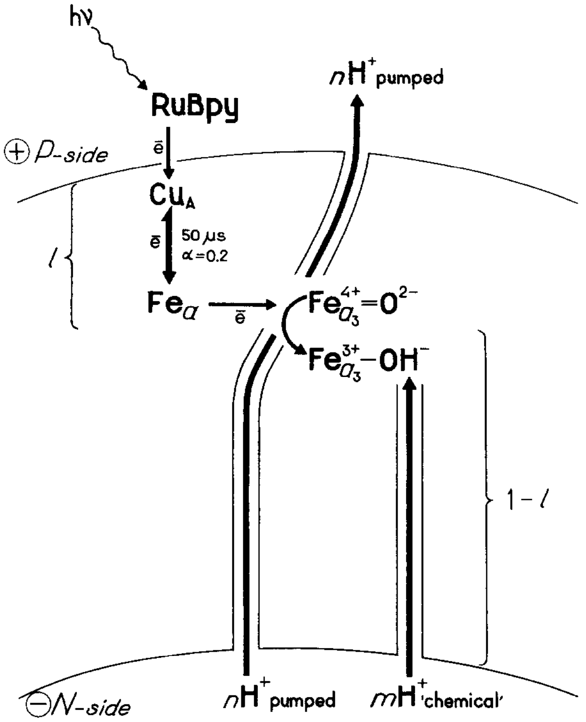

Fig. 3. Electrogenic steps linked to the flash-induced reduction of cytochrome oxidase Compound F. The major electrogenic processes are indicated by thick arrows. The rapid phase is assigned to vectorial electron transfer from C_A, located close to the *P*-side of the membrane [41], to heme *a* immersed deeply into the dielectric barrier [33,41]. The millisecond part of the response is associated with the **F** to **Ox** conversion of heme a_3 linked to reoxidation of heme *a*. Since the protein ligands of heme *a* and those of the binuclear centre metal ions are located at approximately the same distance from the membrane surface [4], e^{-1} transfer from heme *a* to the a_3/Cu_B center per se would not be electrogenic. Accordingly, generation of $\Delta\psi$ coupled to $a_3^{4+}=O^{2-}$ reduction by heme *a* is likely to originate in vectorial proton transfer which may include at least two different processes: (i) vectorial uptake of *m* 'chemical' protons from the *N*-phase required to produce a hydroxyl anion (or water) from the heme a_3^{4+}-bound oxygen, and (ii) transmembrane transfer of *n* protons linked to the reduction of **F** to **Ox** (pumped protons).

value of $\alpha = 0.2$ from our measurements we obtain for the number of protons pumped:

$$n = 2 - 0.5m \qquad (3)$$

if $m = 2$ [2], than $n = 1$, rather than 2 as suggested in [2,13]. However, if $m = 1$, as found in [34,35], then n can be as high as 1.5, directly confirming the conclusion [13] that conversion of **F** to **Ox** is characterized by a charge translocated per e^- stoichiometry higher than 1. Nevertheless, this value is still less than $n = 2$ implied by the scheme suggested in [2]. (Incidentally, a value of $n = 1.7$ was obtained by Wikstrom for the **F** → **Ox** transition experimentally [13] but corrected to 2 with certain assumptions).

We consider this discrepancy significant (note that if $l < 0.5$ or/and $\alpha > 0.2$, which may be the case, n will be even less than 1.5) and would concern two possible meaningful reasons for n value underestimation in our experiments or/and their interpretation:

(i) The **F** state as generated by peroxide addition here or in [25], is heterogeneous (e.g. a mixture of the input and output conformations, or of the protonated and unprotonated states, cf. [25,38]), so that full transmembrane proton pumping is linked to the reduction of only a fraction of the intermediate; this would also account for the biphasic reoxidation of heme *a* associated with the two millisecond electrogenic phases ([25,38], this work).

(ii) Electrogenic contribution of the Cu_A-to-heme *a* vectorial e^--transfer is overestimated. This may be the case if heme *a* reduction by Cu_A were linked to an additional electrogenic process, e.g. uptake of proton(s) from the *N*-phase. The latter possibility may be implied by our early finding [6] confirmed by the Helsinki and, more recently, by the Glynn groups [39,40], that when the binuclear centre is oxidized, heme *a* reduction is linked thermodynamically to protonation of some ionizable group(s), possibly associated with Cu_A [40], from the *N*-aqueous phase.

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