Rapid kinetics of membrane potential generation by cytochrome c oxidase with the photoactive Ru(II)-tris-bipyridyl derivative of cytochrome c as electron donor

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Abstract Yeast iso-1-cytochrome c covalently modified at cysteine-102 with (4-bromomethyl-4'-methylbipyridine)[bis(bipyridine)]Ru^{2+} (Ru-102-Cyt c) has been used as a photoactive electron donor to mitochondrial cytochrome c oxidase (COX) reconstituted into phospholipid vesicles. Rapid kinetics of membrane potential generation by the enzyme following flash-induced photoreduction of Ru-102-Cyt c heme has been measured and compared to photovoltaic responses observed with Ru(II)(bipyridyl)$_3$ (RuBpy) as the photoreductant [D.L. Zaslavsky et al. (1993) FEBS Lett. 336, 389–393]. At low ionic strength, when Ru-102-Cyt c forms a tight electrostatic complex with COX, flash-activation results in a polyphasic electrogenic response corresponding to transfer of a negative charge to the interior of the vesicles. The initial rapid phase is virtually identical to the 50 μs transient observed in the presence of RuBpy as the photoactive electron donor which originates from electrogenic reduction of heme a by Cu_A. Cu_A reduction by Ru-102-Cyt c turns out to be not electrogenic in agreement with the peripheral location of visible copper in the enzyme. A millisecond phase (τ ca. 4 ms) following the 50 μs initial part of the response and associated with vectorial translocation of protons linked to oxygen intermediate interconversion in the binuclear centre, can be resolved both with RuBpy and Ru-102-Cyt c as electron donors; however, this phase is small in the absence of added H_2O_2. In addition to these two transients, the flash-induced electrogenic response in the presence of Ru-102-Cyt c reveals a large slow phase of $\Delta \Psi$ generation not observed with RuBpy. This phase is completely quenched upon inclusion of 100 μM ferricyanide in the medium and originates from a second order reaction of COX with the excess Ru-102-Cyt c^{2+} generated by the flash in a solution.

Key words: Cytochrome oxidase; Proton pumping; Rapid kinetics; Ru-modified cytochrome; Membrane potential; Oxygen intermediate

1. Introduction

Cytochrome c oxidase is a terminal enzyme of the mitochondrial respiratory chain which accepts electrons from cytochrome c and transfers them to molecular oxygen, the latter

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being reduced to water [1]. The enzyme converts the free energy of this strongly exergonic reaction into the energy of the transmembrane difference of the H^+ ion electrochemical potential, $\Delta \mu_{H^+}$ [2,3]. Conversion of energy occurs by the vectorial mechanism i.e. directed across the membrane, and thus electrogenic transfer of protons and electrons [2–4], of which vectorial proton transfer makes the major contribution to membrane energization [1,4,5].

Deciphering the mechanism of the COX-catalysed reaction depends to a significant extent on an ability to resolve the individual steps and intermediates of this complex process. Time-resolved studies of the enzyme oxidation and reduction have been carried out following intramolecular electron distribution with the use of a number of spectroscopic techniques as well as proton release and uptake associated with the electron transfer steps and a number of intermediates have been resolved (reviewed, [1,5–7]).

The rapid kinetics studies on COX fall into the oxidation, reduction and redox equilibrium perturbation experiments. For many years the reductant pulse studies, e.g. reduction by cytochrome c, lagged behind with respect to time resolution since the rapid-mixing techniques used to initiate reduction of COX could not compete with the methods based on flashing off CO widely applied in the O_2 oxidation and perturbation experiments.

However, a number of photoactive potential electron donors for COX have been introduced recently [8–13], of which $Ru(Bpy)_3$ [10] and a family of cytochrome c derivatives singly modified at various amino acid residues with Ru^{2+}-polypyridyl compounds [11–13] have proved to be the most promising. Recent experiments with these donors [10,13], as well as pulse radiolysis studies [14], allowed the resolution of the redox events following single electron injection into COX on a submillisecond time scale.

In a previous paper [15] we took advantage of the RuBpy technique [10] to monitor rapid kinetics of membrane potential generation by COX in phospholipid vesicles following photoinjection of a single electron into the Cu_A centre of the enzyme. Electrogenic phases associated with (i) electron transfer from Cu_A to heme a and (ii) vectorial proton translocation coupled to reoxidation of heme a by the oxoferryl compound of heme a_3 were resolved [15]. However, RuBpy is an artificial electron donor and the driving force for electron transfer from the photoexcited Rubpy to COX is very high. Therefore, as discussed in [10], the possibility of electron transfer route(s) different from the physiological could not be excluded.

Here we describe time-resolved electrogenic reactions of

COX using a photoactive cytochrome c derivative Ru-102-Cyt c (yeast iso-1-cytochrome c labeled covalently with (4-bromo-methyl-4'-methylbipyridine)[bis(bipyridine)]Rus^{2+} at Cys-102) [12]) in which the photoactive group is attached to the single sulfhydryl group (Cys-102) on the back surface of the cytochrome c molecule opposite the heme crevice. Such a location of the Ru label is expected not to interfere with the cytochrome binding to COX, allowing for a normal redox interaction of the modified cytochrome c with the enzyme. Ru-102-Cyt c has been used before in studies of cytochrome c peroxidase [12], however, its reaction with COX has not been investigated.

2. Materials and methods

Ru(Bpy)$_3$Cl$_3$ was purchased from Aldrich. Ru-102-Cyt c was prepared as described in [12]. All other basic reagents and procedures, like the isolation of COX, reconstitution of the enzyme into phospholipid vesicles (COV), association of COV with the collodion film, and electrometric assay of the electrogenic activity of the enzyme, were as described in a previous paper [15]. 10 mM aniline was used as the sacrificial electron donor for the photooxidized Ru. Flash-excitation of the Ru-containing photoactive complexes was made with 15 ns laser flashes delivered from a YAG laser (Quantel 481) operated in a double frequency mode (532 nm).

Computer analysis of the kinetic traces was carried out with the aid of the software package GIM (Graphic Interactive Management) developed by Dr. Alexandre L. Drachev.

3. Results

When collodion film-associated COV are supplemented with 5 μM Ru-102-Cyt c at low ionic strength, a laser flash gives rise to the generation of a membrane potential with a polarity corresponding to the transfer of a positive charge from the inside to the outside of the vesicles (Fig. 1). The overall time-course of the response differs significantly from that observed earlier with RuBpy as the photoreductant [15] by the presence of a major very slow component of $\Delta \Psi$ generation, reaching saturation in about 0.5 s; for comparison the photoelectric response with RuBpy develops fully within ca. 10 ms ([15], Fig. 3 of this paper) and, on the timescale of 1 s, there follows a discharge of the photopotential (not shown).

Inclusion of 100 μM ferricyanide in the reaction buffer completely eliminates the subsecond component of the response, affecting the rapid part only slightly (Fig. 2). The difference between the kinetic traces obtained in the absence and in the presence of ferricyanide (Fig. 2, curve 3) gives an exponent with k_{app} of ca. 0.2 s^{-1}. We have also found that, whereas the rapid part of the COX photoelectric response in the presence of Ru-102-Cyt c, as well as of RuBpy, is fully abolished by increasing the ionic strength of the buffer (100 mM NaCl), the subsecond phase persists, although it decelerates somewhat (data not included).

In Fig. 3 the initial parts of the COX photoelectric responses observed with Ru-102-Cyt c and RuBpy as the electron donors are compared on the same timescale. In the presence of 4 mM H$_2$O$_2$, which converts the enzyme into the oxoferryl state [16], flash-induced injection of an electron into COX from the pre-bound RuBpy gives rise to an electrogenic response (curve 1) showing an initial phase with τ of about 50 μs associated with heme a reduction by Cu$_A$, and a much larger millisecond phase originating from vectorial proton transfer steps linked to oxoferryl intermediate reduction by heme a [15]; the millisecond
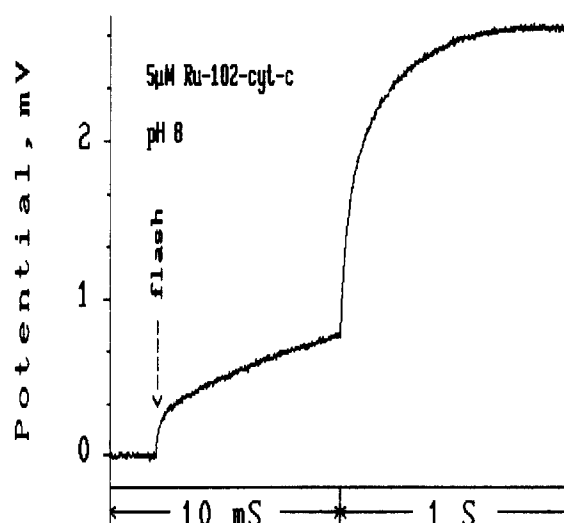

Fig. 1. Flash-induced generation of membrane potential by collodion film-adhered cytochrome oxidase vesicles in the presence of Ru-102-Cyt c. The final reaction buffer contained 5 mM Tris-acetate, pH 8, 10 mM aniline and 5 μM Ru-102-Cyt c. The laser flash is indicated by a vertical arrow.

phase can be further deconvoluted into two components with τ of ca. 1 and 4 ms (not shown, cf. [15]).

In the absence of added H$_2$O$_2$ (curve 2), the same initial 50 μs transient is observed with RuBpy whereas the millisecond phase becomes small. As discussed in [15], aerobic liposome-incorporated COX turns over slowly, oxidizing endogenous reductants present in the phospholipids; this result in a small, variable steady-state fraction of the oxoferryl and/or peroxy states generated, reduction of which gives rise to the residual millisecond phase; notably, this phase in curve 2 of Fig. 3 reveals the presence of the same two components (ca. 1 and 4 ms) as the major part of curve 1 (not shown).

A photoelectric response with Ru-102-Cyt c in the absence of added H$_2$O$_2$ is given in Fig. 3 by curve 3. The kinetics of the initial part of the curve is almost indistinguishable from that observed in the experiments with RuBpy*, given that the responses are normalized to the amplitude of the 50 μs phase. (Generally, the photoelectric responses with Ru-102-Cyt c were much smaller than with Rubpy, which is mainly due to a rather poor quantum yield of heme c photoreduction with this particular cytochrome c derivative.) Computer analysis shows that the slower part of trace 3 contains a small millisecond phase, similar to that in curve 2, which is obscured by the major slow reaction discussed above (cf. Fig. 2).

In the preliminary experiments with Ru-102-Cyt c in the presence of added H$_2$O$_2$, we have encountered artifacts (color development and generation of a voltage across the collodion film in the dark) associated most probably with the peroxidase

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*The observed τ for the initial phase of the photoelectric response with Ru-102-Cyt c was consistently slightly higher than with RuBpy (about 70 μs vs. 50 μs). This is a mere consequence of the equilibrium distribution of the photoinjected electron between heme c and Cu$_A$ in contrast to irreversible reduction of Cu$_A$ by RuBpy. Simple formal kinetics considerations taking into account E_m values of heme c[12] and Cu$_A$ [1] convert the τ_{obs} of 70 μs to the genuine value of ca. 50 μs for the elementary step of heme a reduction by Cu$_A$.
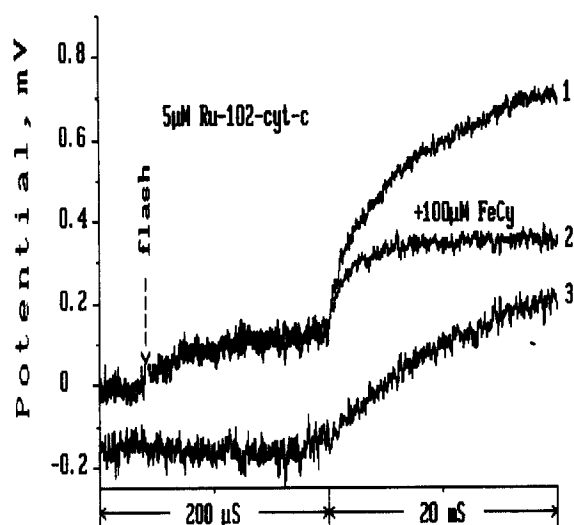
Fig. 2. Effect of ferricyanide on the kinetics of the flash-induced $\Delta\Psi$ generation with Ru-102-Cyt c. Basic conditions, as in Fig. 1. Trace 1 was recorded, 100 μM ferricyanide was then added and in ca. 5 min the sample was flashed again to obtain trace 2. Trace 3 gives the difference between trace 2 and trace 1. The curves are given as recorded without normalization.

activity of the modified cytochrome c (aniline peroxidation). Therefore, detailed comparison of the electrogenic reactions of COX associated with the single electron reduction of the oxo-ferryl state of the enzyme by RuBpy and Ru-102-Cyt c has not yet been possible.

4. Discussion

It was shown recently by Millett and co-workers [12] that when the heme iron of cytochrome c bound to COX electrostatically at low ionic strength is photoreduced on a submicrosecond scale by Ru^{2+}-polypiridyl complex attached covalently at various positions on the cytochrome globule, there follows rapid transfer of the electron, first to Cu$_A$ which is over in less than 10 μs (an apparatus dead-time in [12]), and then from Cu$_A$ to heme a, with τ of about 50 μs. Very similar rates of heme a reduction have been reported in [8,10,14] (and cf. [17]). The kinetics of the microsecond part of the COX photoelectric response observed in this work with Ru-102-Cyt c, as well as with RuBpy ([15], this work), is in excellent agreement with the time–course of heme a photoreduction, as measured optically for other cytochrome c derivatives in [12]. This shows directly for the first time that electron transfer from cytochrome c to heme a is indeed electrogenic, as proposed by Mitchell many years ago [2,18]. Moreover, it is clear from the present data that $\Delta\Psi$ generation linked to heme a reduction by cytochrome c is associated exclusively, or almost exclusively, with electron transfer from Cu$_A$ to heme a, whereas the reduction of Cu$_A$ by cytochrome c does not give rise to any discernible electrogenic reaction, which would have appearde as a rapid initial jump in the ≈ 50 μs phase of the photovoltaic curve. Electron transfer from heme c to Cu$_A$ notbeing electrogenic is in line with the results of the equilibrium measurements (see [19] and refs. therein) and points to a peripheral location of the visible copper in the enzyme.

It is noteworthy that the initial electrogenic phase observed

with Ru-102-Cyt c and RuBpy are practically superimposable (cf. footnote*), which indicates that the electron is transferred from RuBpy to heme a by the same route as in the case of cytochrome c, the physiological donor. This gives further credence to our previous results on the rapid kinetics of $\Delta\Psi$ generation by COX obtained with RuBpy as the photoreductant [15].

As to the very slow (subsecond) part of the photoinduced electric response observed with Ru-102-Cyt c but not with RuBpy, there are good reasons to attribute this phase to the second order reaction between COX and the excess of the reduced cytochrome c generated by the flash in the solution (cf. [12]). In particular, this phase is completely abolished by 100 μM ferricyanide (Fig. 1). If we consider a rate constant of ca. 10^7 M$^{-1}\cdot$s^{-1} for the second order reaction between ferricyanide and ferrocytochrome c in solution [20], the characteristic time expected for oxidation of the excess photoreduced Ru-102-Cyt c will be in the range of 1 ms, which is sufficient for virtually complete elimination of the subsecond electrogenic phase.

The electrogenesis associated with the ferricyanide-sensitive second-order reaction originates most probably from a multiple turnover oxidation of ferrocytochrome c by COX under aerobic conditions. It has to be pointed out that the amount of the collodion film-adhered COX in our experiments, when divided by the electrometric cell volume, converts to a concentration of about 10^{-15}–10^{16} M and therefore, even very low concentrations of ferrocytochrome c photogenerated in the solution (submicromolar or nanomolar) are still in huge molar excess over cytochrome oxidase.

As to the millisecond part of the photoelectric response which reflects intramolecular electron/proton transfer steps associated with reoxidation of the photoreduced heme a and concomitant reduction of the oxygen intermediates in the binuclear centre [15], it has been observed with both RuBpy or Ru-102-Cyt c (Figs. 2 and 3) and no major difference in the
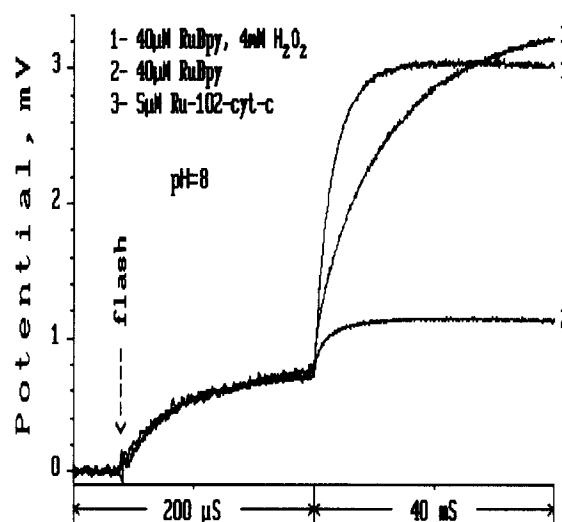

Fig. 3. Comparison of the kinetics of membrane potential generation by COX with RuBpy (1,2) and Ru-102-Cyt c (3) as photoactive electron donors. The electrometric cell contains 5 mM Tris-acetate buffer pH 8, 10 mM aniline and either 40 μM RuBpy (traces 1 and 2) or 5 μM Ru-102-Cyt c. In the case of trace 1, 4 mM H$_2$O$_2$ is also present. The traces obtained with RuBpy and Ru-102-Cyt c have been normalized to the amplitude of the 50 μs phase of the response; the absolute values on the ordinate scale refer to the experiments with RuBpy (curves 1,2).

kinetics of this phase between the two donors were revealed so far. Unfortunately, we are not yet in a position to provide a detailed comparison of this, perhaps most interesting, part of the response between Ru-102-cyt *c* and RuBpy because, in the absence of added peroxide, the amplitude of this phase is rather small and variable, and it has not been possible to carry out experiments with added H_2O_2, i.e. starting with the oxoferryl of peroxy state as the major form of the enzyme, with Ru-102-Cyt *c*.

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