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Biochimica et Biophysica Acta 1318 (1997) 6–10

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

Rapid report

Translocation of electrical charge during a single turnover of cytochrome-*c* oxidase

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Received 18 September 1996; accepted 7 October 1996

Abstract

In cell respiration, cytochrome-*c* oxidase utilizes electrons from catabolism to reduce O_2 to water. Energy is conserved as an electrochemical proton gradient across the mitochondrial membrane, which drives the synthesis of ATP. Electrical charge translocation during the reaction of the reduced enzyme with O_2 takes place in two phases of identical amplitude. The first phase ($\tau_1 = 0.2$ ms) occurs after an initial lag, and appears to correspond to the transition from a peroxy to a ferryl intermediate in the oxygen chemistry. The second phase ($\tau_2 = 2.6$ ms) matches the transition from the ferryl intermediate to the oxidised enzyme. These findings define the kinetic linkage between the chemistry and the major events of proton pumping by the enzyme.

Keywords: Cytochrome-*c* oxidase; Electrical charge; Cell respiration

There are two aspects to the mechanism by which the cytochrome oxidase reaction generates an electrochemical proton gradient (Fig. 1). Half of the energy conservation is a direct consequence of the vectorial nature of the oxygen chemistry itself; four electrons enter the oxygen reduction site from the outer aqueous side of the membrane, while four protons are taken from the inner side. Thus reduction of one molecule of O_2 to two water molecules is equivalent to moving four electrical charges across the membrane. In addition to this, the enzyme is an electrogenic proton pump; for every O_2 reduced to water, an additional four protons are translocated from the inside of the membrane to the outside. This proton pumping process appears to be coupled directly to the oxygen chemistry [1], but very little is known about its mechanism (for reviews, see Refs. [2,3]).

The proton pumping function was discovered in experiments where the enzyme was allowed to turn over a number of times with observation of acidification and alkalinisation in the external and internal aqueous phases, respectively [4–6]. However, insight into the mechanism requires time-resolved proton translocation measurements during a single enzyme turnover. Rapid kinetic methods have been developed to study the cytochrome-*c* oxidase reaction, but fast measurements of pH [6,7] and of charge translocation (membrane potential, $\Delta\psi$; [8]) have only recently begun to emerge. We now report direct measurements of $\Delta\psi$ during the reaction of the fully reduced

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PII S0005-2728(96)00147-8

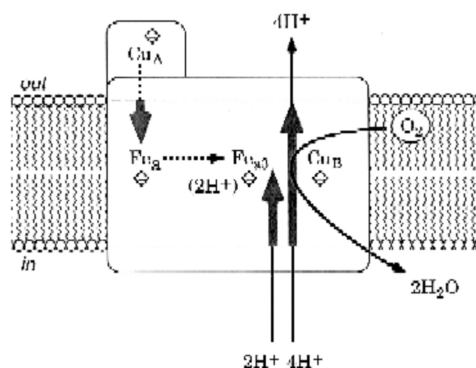

Fig. 1. An illustration of cytochrome-*c* oxidase in the membrane. Cu_A is the first electron acceptor site in the enzyme. From Cu_A electrons are transferred via a low-spin heme (Fe_a) to an oxygen reduction site consisting of a heme (Fe_{a3}) and a copper (Cu_B). The events which take place during the reaction of the fully reduced enzyme with oxygen are illustrated schematically by arrows. Diamonds indicate the initial location of the four electrons which eventually go into water; dashed arrows denote electron transfers; solid straight arrows denote proton movements; large grey-scale arrows denote charge movements which contribute to $\Delta\psi$; dashed horizontal lines indicate the limits of the dielectric within the enzyme. The reduced enzyme has already taken up two protons [23], shown in parenthesis. Although not specified in the scheme, one or both of these may contribute to the 4 H^+ released on the outside, or may be consumed in water formation.

membrane-incorporated enzyme with oxygen, together with parallel optical measurements which monitor the chemistry.

The fully reduced enzyme holds four reducing equivalents – exactly the number required to reduce O_2 to two water molecules. Hence, this reaction represents the complete oxidative part of the enzyme cycle. One reducing equivalent is initially associated with each of the redox-active metal centers in the enzyme (Fig. 1). In order to observe $\Delta\psi$, the enzyme must be incorporated in a closed lipid bilayer membrane; therefore isolated cytochrome-*c* oxidase was reconstituted into phospholipid vesicles [9]. The method of direct, time-resolved electrical measurements is described in Fig. 2 (cf. Refs. [8,10,11]). Since the reaction of the enzyme with oxygen takes place too rapidly to be resolved by usual mixing methods (but see Ref. [12]), these experiments employ a method in which CO ‘cages’ the reduced enzyme so that the reaction with O_2 can be initiated by a flash of light [13].

Fig. 3 shows the time course of the reaction with O_2, monitored both in the electrical domain (frames B and D), and by the conventional optical method (frames A and C). The initial steps of the reaction can be followed most clearly from the optical measurements at 445 nm, which corresponds to the absorbance maximum of the reduced heme groups of the unliganded enzyme. At the beginning, the enzyme is fully reduced with CO bound at the oxygen-binding heme. O_2 has been mixed into the sample, but cannot bind because the site is blocked by the CO. The experiment begins with a laser flash, which breaks the Fe_{a3}-CO bond and produces the sharp

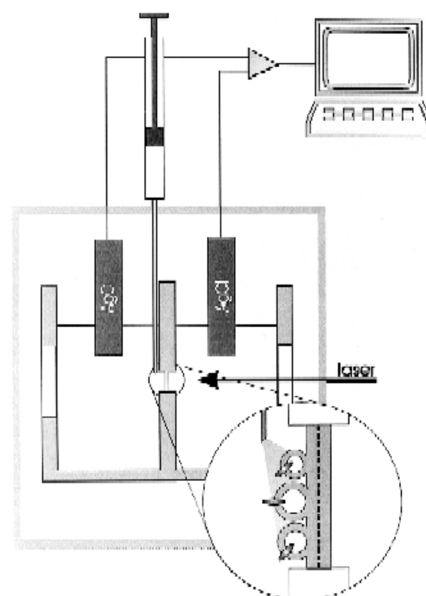

Fig. 2. Diagram of the membrane electrical measurement system. This is based on a method originally developed by Drachev and co-workers [10,11]. In the present system, electrodes record the voltage across a measuring membrane consisting of a lipid-impregnated teflon mesh. Proteoliposomes in one compartment are forced to fuse to this membrane by addition of Ca^{2+}. The amplitude of the voltage across the measuring membrane will increase proportionally to that of the vesicle membranes allowing the kinetics of charge translocation to be followed. The apparatus is enclosed in a gas-tight box which is first purged with argon and then filled with CO. The liquid compartments contain glucose oxidase and glucose to remove any remaining oxygen. A syringe is used to inject oxygen saturated buffer a fraction of a second before the laser flash starts the reaction. (A micro switch on the syringe triggers the event cascade.) The jet is directed at the measuring membrane (a 4 mm circle), producing a local high concentration of oxygen for the time of the reaction.

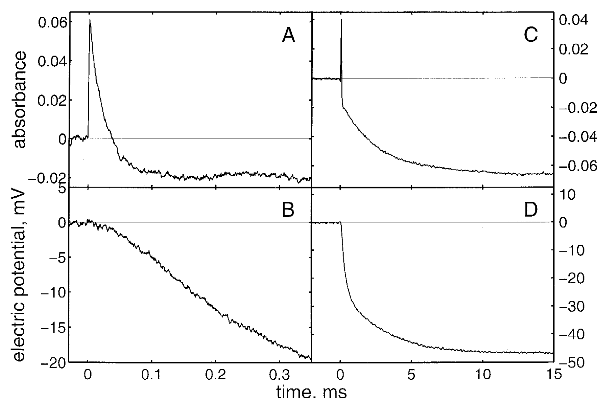

Fig. 3. The reaction of fully reduced cytochrome-*c* oxidase in proteoliposomes with oxygen. A and C, optical absorption at 445 nm (methods described in Ref. [31]); B and D, direct electrometric measurements (voltage on membrane; Refs. [8,10,11]). A,C; cytochrome-*c* oxidase, 0.9 μM; oxygen, 1 mM; medium: pH 7.0 MOPS 10 mM, sucrose 100 mM; catalase, 75 μg/ml (concentrations after mixing). Proteoliposomes (9; typical respiratory control ratios between 5 and 8) were made anaerobic, reduced by dithionite (0.5 mM before mixing) and then place under 1 atm CO. B,D, Proteoliposomes attached themselves to the membrane during a 2-h incubation in 12.5 mM $CaCl_2$, pH 7 MOPS 10 mM. This medium, and the remaining unattached vesicles, were then replaced using pH 7 MOPS 10 mM and the following additions made: glucose oxidase 0.12 mg/ml; catalase, 75 μg/ml; glucose, 150 mM; N,N,N',N'-tetramethyl-p-phenylenediamine, 50 μM, CO 1 atm (see Fig. 2).

increase in absorbance at $t = 0$. This is followed by a large decrease ($\tau = 0.026$ ms), which is due to binding of O_2 and its initial reduction, together with the oxidation of both hemes [14–17]. At the end of this phase, the oxygen reduction site thus contains three of the four reducing equivalents in the enzyme, and has been proposed to form a ferric peroxy state [1,2,17,18]. Now, an apparently uneventful absorption phase follows, during which it is thought that the peroxy intermediate is converted to a ferryl state [19,20], concomitant with formation of the first water molecule. At approximately the same time the remaining (fourth) electron, originally located on Cu_A, redistributes between Fe_a and Cu_A ([21]; cf. Fig. 1). Finally, there is a slow decrease in absorbance as this fourth electron finds its way to the oxygen reduction site where it reduces the ferryl intermediate to a ferric state thus resulting in the fully oxidised enzyme. A second water molecule is produced and the reaction ends. This final phase is slower here than is usually observed in the detergent-solubilized enzyme ($\tau = 2.6$ ms compared to 1.2 ms, [15]). This is presumably a manifestation of 'respiratory control', viz. retardation of enzyme turnover by the electrochemical proton gradient.

Before discussing the electrometric results, it should be emphasized that the main energy-requiring reaction steps of proton pumping (i.e., translocation of four charges across the dielectric; Fig. 1) have been shown to be associated with the reactions of the reduced enzyme with O_2 studied here [1]. Additional, though comparatively minor contributions to $\Delta\psi$ are expected from two sources (see Fig. 1), viz. transfer of one electron from Cu_A to Fe_a (one charge across one half of the dielectric; [8,22]), and net consumption of two protons from the inside (two charges across one half of the dielectric; Refs. [23,24]). Hence, proton pumping is by far the major contributor to electrogenicity in the overall reaction (ca. 75%). Note, however, that the electrogenic steps in the mechanism of proton pumping might result either from movement of protons, and/or from movement of negatively charged proton carriers in the opposite direction. These mechanistic alternatives are obviously indistinguishable by the electrometric technique.

By contrast to the optical data, in the electrical domain CO photolysis, oxygen binding and the first heme oxidation phase are almost silent (Fig. 3B). Following this initial lag, $\Delta\psi$ develops in two phases of almost identical amplitude with time constants of 0.2 and 2.9 ms (Fig. 3B,D). The initial lag is not unreasonable. CO photolysis and dioxygen binding involve electrically neutral molecules. The initial heme oxidation phase includes electron transfer from Fe_a to the oxygen reduction site [14–17], but this is not expected to be an electrogenic event, since structural studies [25–27] have shown that the two heme groups lie at about the same depth relative to the membrane (Fig. 1). $\Delta\psi$ begins to grow only at the end of the initial phase of heme oxidation. Since the first electrogenic phase ($\tau_1 = 0.2$ ms) coincides with the plateau in the optical data, it apparently occurs at the time of scission of the O-O bond in the conversion of the peroxy to the ferryl intermediate, and the formation of the first molecule of water. The redistribution of electrons between Cu_A and Fe_a also occurs at this step (see above), and should hence make a small contribution to the first electrogenic phase.

The slower electrogenic phase has a time constant ($\tau_2 = 2.9$ ms), very similar to that of the final phase in the optical measurement ($\tau = 2.6$ ms). This suggests that they are both connected to the conversion of the ferryl intermediate to the oxidised enzyme and a second molecule of water. Here again, a small electrogenic contribution from electron transfer is expected, since about one half of the electron equivalents originates in Cu_A (the other half in Fe_a).

Oliveberg et al. [7] measured net proton uptake, i.e., the protons used for formation of water, by the detergent-solubilized enzyme during its oxidation by O_2. The kinetics were very similar to those of $\Delta\psi$ here, with a similar lag and two proton uptake phases of almost identical amplitude. Hence, the two electrogenic phases here closely follow the kinetics of proton uptake in the chemistry of water formation. This indicates that the movement of pumped protons is intimately tied to the uptake of protons for the oxygen chemistry.

Zaslavsky et al. [8] used a related electrometric technique, but studied only the conversion of the ferryl intermediate to the oxidised enzyme, i.e., the final step in the four-electron process studied here. In a reaction originally devised by Nilsson [28], they first added H_2O_2 to the oxidised enzyme to generate the ferryl intermediate, and then used a photoreductant to inject a single electron into Cu_A. During the reaction they monitored three phases of $\Delta\psi$ generation with time constants of 0.044, 1.2 and 4.9 ms. The fastest phase, which took place without a lag, was assigned to the electrogenic electron transfer from Cu_A to Fe_a. In contrast to the present approach, when the reaction is triggered in this way, the Cu_A to Fe_a electron transfer emerges as a distinct kinetic process. The subsequent millisecond phases were assigned to conversion of the ferryl state to the oxidised enzyme [8], and are comparable to the final $\Delta\psi$ phase in our reaction ($\tau_2 = 2.6$ ms).

Studies of proton ejection from enzyme-containing vesicles using pH dyes [6,7] have found proton ejection ($\tau \sim 1$ ms) corresponding approximately to our second electrical phase, but nothing that would correspond to the faster phase. It is possible, therefore, that the actual release of the pumped protons into the outer aqueous phase is delayed, even though the electrical charge has been carried across the dielectric. This may well be analogous to the case described for bacteriorhodopsin [29], where the release of pumped protons from the surface into the aqueous medium is considerably delayed relative to the catalytic photochemistry. However, the present data show that the main energetic (electrogenic) events coincide kinetically with the peroxide to ferryl, and the ferryl to oxidised transitions of the enzyme. The charge translocation observed in both these steps have minor contributions from electrogenic electron transfers (Cu_A to Fe_a), and from uptake of protons to generate water; it must mainly be due to proton pumping across the dielectric. The nearly equal size of $\Delta\psi$ in the two phases is consistent with a proton pump mechanism that cycles twice during the reduction of O_2, in intimate linkage with the uptake of protons to form water [1,24,30].

This work was supported by the Finnish Academy, the Sigrid Jusèlius Foundation and the Biocentrum Helsinki. We thank Nikolai Belevich for help in design and construction of instruments, Martti Heikkinen for mechanical shop work, and Katja Sissi-Mansour for laboratory assistance.

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