Volume 318, number 2, 134–138 FEBS 12161 March 1993

Light-induced structural changes in cytochrome *c* oxidase

Measurements of electrogenic events and absorbance changes

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Received 24 December 1992

We have investigated flash-induced electrogenic events and absorbance changes in cytochrome *c* oxidase in the absence of dioxygen and carbon monoxide. Electrogenic events were studied using a Teflon-bound layer of cytochrome *c* oxidase oriented in a phospholipid monolayer. Absorbance changes were observed exclusively in partly reduced cytochrome *c* oxidase; the largest changes were found in the one-electron-reduced species. Electrogenic events were detected in all reduction states of the enzyme. Both types of experiments displayed a rapid (< 0.5 μs) event followed by a biphasic relaxation. The time constants of the relaxation were 6 ± 2 μs and 70 ± 10 μs in the electrogenicity, and 9 ± 3 μs and 81 ± 6 μs in the absorbance changes (at ~ 22 °C). The kinetic absorbance difference spectrum was consistent with that of reduced minus oxidized haem. The experimental results are discussed in terms of structural changes in the vicinity of cytochrome a_3. These changes may play an important role in all studies that involve flash photolysis of cytochrome *c* oxidase–ligand complexes.

Cytochrome *c* oxidase; Flash photolysis; Ligand; Electron gating; Electron transfer

1. INTRODUCTION

Cytochrome *c* oxidase (COX) catalyses a vectorial reaction in which electron transfer from cytochrome *c* to dioxygen is coupled to proton transfer from the matrix to the cytosol side of the inner mitochondrial membrane [1]. The enzyme binds four redox-active metal sites: two copper sites, Cu_A and Cu_B, and two haem groups, cytochromes *a* and a_3. Electrons from cytochrome *c* are first transferred to Cu_A and cytochrome *a*, and then to the a_3–Cu_B pair, where the oxygen reduction takes place.

The electron current through COX in the experimentally available pH range is 100–1,000 electrons·s^{-1} [2,3]. However, many intramolecular electron-transfer reactions are much faster. To study these reactions, techniques have been developed which are based on photodissociation of carbon monoxide from partly or fully reduced COX. For example, flash photolysis of CO from the partly reduced enzyme under anaerobic conditions allows time-resolved studies of intramolecular electron-transfer reactions [4]. Under aerobic conditions CO dissociation is followed by binding of dioxygen, which allows time-resolved studies of the reduction of O_2 by COX [5]. A similar approach has also been used to characterize the primary steps in the oxygen reduction; flash photolysis of the intermediate states formed after onset of the reaction has revealed photolabile states formed during the first ~ 100 μs of the reaction [6,7].

In this study we have measured flash-induced electrogenic events and absorbance changes in COX in the absence of CO and O_2. The electrogenicity was studied in COX incorporated in a phospholipid monolayer. Our results indicate structural changes in COX, which most likely occur in all types of flash-induced studies and may thus affect the interpretation of experimental results.

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Abbreviations: COX, cytochrome *c* oxidase (ferrocytochrome *c*:oxygen oxidoreductase, EC 1.9.3.1).

2. MATERIALS AND METHODS

2.1. *Materials*

Cytochrome *c* oxidase from bovine heart was purified as described previously [8] and stored under liquid nitrogen until used. Prior to experiments the enzyme was diluted in 100 mM Hepes-KOH (Boehringer), pH 7.4 and 0.1% (w/v) dodecyl maltoside (Boehringer). All other chemicals were of the purest grade available.

2.2. *Sample preparation*

Peroxy (P state) and ferryl (F state) intermediates of COX [9] were prepared by reacting the two- or three-electron-reduced enzyme with dioxygen or by titration with $H_2O_2$to the spectral characteristics [10] of the desired intermediate. The one-electron-reduced enzyme (E state) was prepared by reduction of the ferryl intermediate by carbon monoxide as described [11], after which CO was replaced by N_2.

To prepare lipid vesicles, soybean lecithin (Sigma, MO, USA), puri-

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fied as described in [12], was dispersed to 10 mg/ml in a solution containing 10 mM HEPES-KOH at pH 7.4 and 10 mM KCl, and vortexed for ~ 5 min. The lipid solution was sonicated (Bronson Sonicator, model B-12, CT, USA) for ~ 10 min until the solution was optically clear as evidenced from the 650 nm absorbance. The vesicle solution was supplemented with 35 μl COX (from a 300 μM solution in 100 mM HEPES-KOH, pH 7.4, 0.1% (w/v) dodecyl maltoside), sonicated for 1 min, and then incubated at +4°C for 15 h.

2.3. *Electrical measurements*

The experimental set-up was similar to the one described previously [13–15]; it is shown schematically in the inset of Fig. 1. The technique was first described by Trissl et al. [16]. A Teflon chamber consisting of two aqueous compartments was separated by a 12 μm Teflon film (area ~ 0.3 cm^2). The vesicle solution (see above) was diluted 1:10 in 10 mM HEPES-KOH at pH 7.4, 10 mM KCl and 10 mM $CaCl_2$ and added to one of the cell compartments to a level just below the Teflon film. In the presence of Ca^{2+} the vesicles break and a monolayer is formed at the surface. The liquid level was raised slowly past the Teflon film allowing the monolayer to attach to the Teflon. The other cell compartment contained an electrically conducting solution.

Light-induced voltage changes were measured across Ag/AgCl or Pt electrodes, shielded from actinic light, immersed in the cell compartments. The voltage changes were measured using an operational amplifier (OP128, Burr-Brown).

2.4. *Optical measurements*

The spectrophotometer used was modified from a design described previously [17,18]. The monitoring beam, provided by a 250 W halogen lamp, was filtered through a heat filter and passed through a double monochromator (Oriel, CT, USA). To increase the signal-to-noise ratio interference filters (bandwidth 5 nm) were used at 445 nm and 605 nm. Intensity changes were monitored with a photomultiplier tube (Hamamatsu R269 or R712) connected to a current to voltage converter (Hamamatsu C1053, bandwidth 3 MHz).

2.5. *Light source*

Actinic illumination was provided by an Nd-YAG pulsed laser (Quantel, YG570) at 532 nm. The pulse width was ~ 10 ns and the energy was ~ 100 mJ.

2.6. *Experimental procedures*

Voltages from both experimental set-ups were amplified using a preamplifier with a variable time constant (Stanford Research Systems, model SR560, CA, USA) and recorded on a digital oscilloscope (Nicolet, model 490, WI, USA). Time constants (τ, time to reach 1/e of the amplitude at time = 0) were determined using a Nelder-Mead simplex algorithm in the Matlab software (Math Works, MA, USA).

Errors (S.D.) in time constants and amplitudes are based on five independent experiments from which they were determined.

3. RESULTS

3.1. *Electrogenicity*

Following pulsed illumination of cytochrome *c* oxidase (COX) oriented in the phospholipid monolayer we observed a rapid (< 100 ns) increase in voltage followed by a decrease (relaxation) with time constants 6 ± 2 μs and 70 ± 10 μs (Fig. 1). As a control we applied the COX-containing monolayer on the opposite side of the Teflon film. As expected, this resulted in voltage changes with an opposite sign compared to those shown in Fig. 1.

The observed voltage changes were similar in both reduced and oxidized COX, which shows that they were not associated with electron transfer. The sign of the initial increase in voltage is consistent with a negative charge moving in the direction from the solution towards the Teflon film (see inset of Fig. 1) or a positive charge moving in the opposite direction.

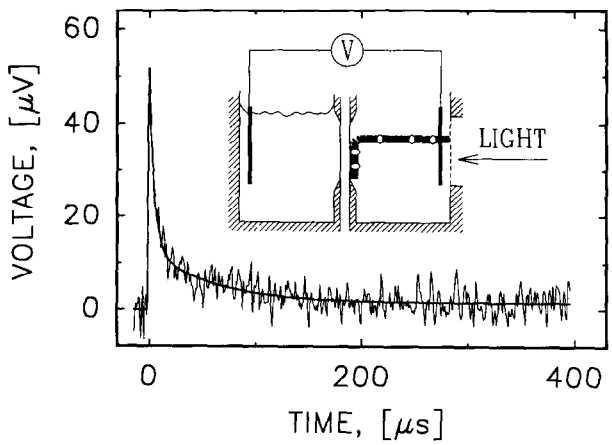

Fig. 1. Voltage changes following pulsed illumination (at time = 0) of cytochrome *c* oxidase oriented in a phospholipid monolayer. The solid line is a least-square fit with a sum of two exponential functions with time constants 6 ± 2 μs (~ 84% of the total amplitude) and 70 ± 10 μs. The inset shows a schematic representation of the experimental set-up used to measure electrogenic events in cytochrome *c* oxidase. The cell chamber was made of Teflon. It contained two compartments separated with a 12 μm Teflon film. The Teflon-bound cytochrome *c* oxidase–lipid monolayer is shown in the right-hand side compartment. Voltage changes were measured across electrodes immersed in the cell compartments. Conditions: 10 mM HEPES-KOH, pH 7.4, 10 mM KCl, 10 mM $CaCl_2$. The temperature was 23 ± 1°C.

3.2. *Absorbance changes*

To characterize further the observed electrogenic events we measured absorbance changes following pulsed illumination of COX. The fully oxidized* COX did not display any measurable absorbance changes. To study flash-induced events in COX reduced to different degrees we added 3.0 mM sodium ascorbate to oxidized COX under a N_2 atmosphere. The amount was sufficient to fully reduce COX within ~ 1 h. Flash-induced absorbance changes were measured at different times after the addition of ascorbate. During this time COX goes through different degrees of reduction. In the partly reduced COX we observed a rapid (< 0.5 μs) increase in absorbance, consistent with reduction of haem, followed by a decrease (relaxation) with time constants 9 ± 3 μs and 81 ± 6 μs to reach the level be-

*After purification a small population of COX was found to be partly reduced (on average ≤ 1 electron per COX). To obtain fully oxidized COX the enzyme solution was titrated with ferricyanide monitoring the absorbance at 605 nm. Initially the absorbance decreased and stabilized then at a level which defined the fully oxidized COX.

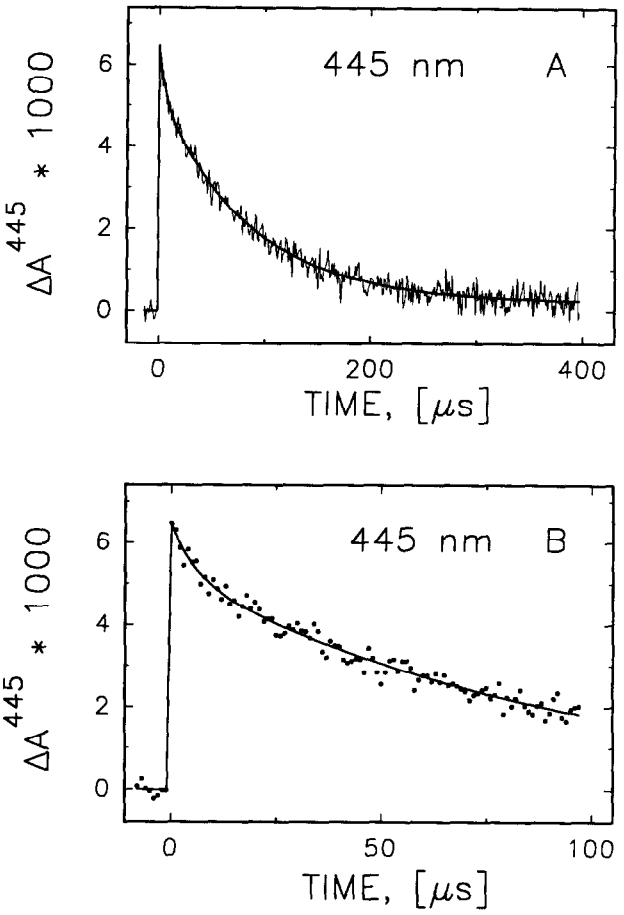

Fig. 2. (A) Absorbance changes following pulsed illumination (at time = 0) of partly reduced cytochrome *c* oxidase under N_2 atmosphere. (B) The absorbance changes on an expanded time scale (the digitized data points are shown). The solid line is a least-square fit with a sum of two exponential functions with time constants 9 ± 3 μs (~ 23% of the total amplitude) and 81 ± 6 μs. The reduction level of the enzyme at the time of the experiment corresponded to ~ 25% of the reduced minus oxidized absorbance difference at 445 nm and at 605 nm. Conditions: 100 mM HEPES-KOH, pH 7.4, 0.1% dodecyl maltoside, 10 μM cytochrome *c* oxidase, 3 mM ascorbate. The temperature was 22 ± 1°C.

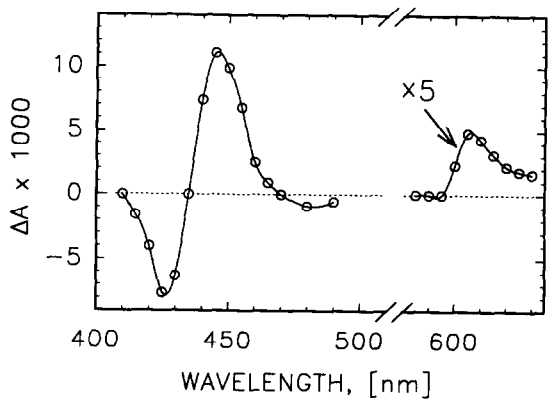

Fig. 3. Kinetic absorbance difference spectrum of the one-electron-reduced COX. The absorbance change at each wavelength is the difference in the absorbance immediately after the flash and the absorbance immediately before the flash (see Fig. 2). Conditions were the same as in Fig. 2, except that no ascorbate was present.

fore the flash (see Fig. 2). The amplitude of the rapid absorbance change immediately after the flash first increased with increased reduction level of COX to reach a maximum when the enzyme was reduced to ~ 25%* (Fig. 2). After this point the amplitude of the flash-induced absorbance change decreased with increasing reduction level of COX to disappear when the enzyme was reduced to ~ 50%.

In addition, we investigated flash-induced events in one-electron-reduced COX (E state). Following pulsed illumination we observed absorbance changes similar to those shown in Fig. 2 but with ~ 2 times larger amplitude. This indicates that the largest absorbance changes are observed in the one-electron-reduced COX. In the ascorbate-reduced enzyme (see above), at any given time after addition of ascorbate, there is a mixture of states with different number of electrons per COX. Therefore, the one-electron-reduced species constitutes only a fraction of COX, which explains why the amplitude of the light-induced absorbance changes in the E state COX was larger than the maximum change in the ascorbate-reduced COX.

Fig. 3 shows a kinetic absorbance difference spectrum* of the absorbance changes observed after pulsed illumination of the one-electron-reduced COX. The spectrum resembles that of reduced minus oxidized haem. The ratio of the absorbance changes at 445 nm and at 605 nm, $\Delta A^{445}/\Delta A^{605}$, was 11 ± 2.

After addition of 20 mM cyanide, which binds to the a_3-Cu_B site [19,20], no detectable absorbance changes were observed following pulsed illumination, which indicates that this site is involved in the reaction.

3.3. *Photolability of oxygen intermediates*

Other results have shown that the transient states formed during the first ~ 100 μs of the reduction of dioxygen by COX are photolabile [6,7]. Therefore, we also investigated the photolability of the stabile peroxy (P state) and ferryl (F state) oxygen intermediates of COX (see Materials and Methods). At pH 7.4 neither of the two forms displayed any spectral changes following pulsed illumination.

*The absorbance at 445 nm and 605 nm increased by ~ 25% of the total reduced minus oxidized difference at each wavelength.

*The difference in absorbance immediately after the flash and immediately before the flash at different wavelengths.

4. DISCUSSION

We have measured flash-induced electrogenic events and absorbance changes in cytochrome *c* oxidase (COX) under N_2 atmosphere reduced with the equivalent of 0–4 electrons. Electrogenic events were observed in all reduction states of COX. Absorbance changes were only observed in partly reduced COX; the largest amplitude was observed in one-electron-reduced COX. The characteristic time constants of both the flash-induced electrogenic events and the absorbance changes in partly reduced COX were, within the experimental error, the same; a rapid (< 0.5 μs) event was followed by a relaxation with time constants of 6–8 μs and 75–80 μs. This suggests that the same flash-induced changes were observed. The kinetic absorbance difference spectrum was consistent with that of reduced minus oxidized haem. In addition, the observed absorbance changes were quenched by cyanide which binds to cytochrome a_3. Consequently, we attribute the observed flash-induced events in COX to cytochrome a_3 or the immediate environment of this centre.

The observed electrogenic events indicate structural changes in COX following pulsed illumination. Recently, Woodruff et al. [21] demonstrated structural changes at the a_3–Cu_B site after flash photolysis of CO from COX. In addition, they suggested the existence of a ligand which may bind to either cytochrome a_3 or Cu_B. Even though CO was not present in our experiments the observed electrogenic events may reflect structural changes as a consequence of the light excitation of, for example, cytochrome a_3.

Fig. 4 shows a schematic diagram, based on the scheme of Woodruff et al. [21], which models our experimental results. In the model, L is a ligand which may bind to either cytochrome a_3 or Cu_B. Following pulsed illumination the proximal histidine of cytochrome a_3 dissociates and is replaced by L. As a consequence, an electron is transferred from Cu_B^+ to cytochrome a_3^{3+}. In 6–8 μs the histidine rebinds, L dissociates, and the electron is transferred back to Cu_B^{2+} with a time constant of 75–80 μs.

The model conveniently explains the observed electrogenic events and absorbance changes; the structural changes are electrogenic and occur in all reduction states of COX. Only in partly reduced COX can these structural changes be associated with electron transfer, which explains why absorbance changes were observed in the partly reduced but not in the fully oxidized and the fully reduced COX. The presence of cyanide stabilises oxidized cytochrome a_3 and impedes the electron transfer, which explains why no absorbance changes were observed in the presence of cyanide.

According to the model the observed absorbance changes are primarily due to the reduction of cytochrome a_3^{3+}, which occurs in the subpopulation of COX in which cytochrome a_3 is oxidized and Cu_B is reduced. Since in the one-electron-reduced COX the electron is distributed between the metal centres [22], only a fraction of COX produces absorbance changes. This explains why the observed absorbance changes are small compared to those expected from the extinction coefficient of reduced minus oxidized cytochrome a_3. In addition, the actinic flashes (see Materials and Methods) were not saturating, which also reduces the observed absorbance changes. Moreover, the extinction coeffi-

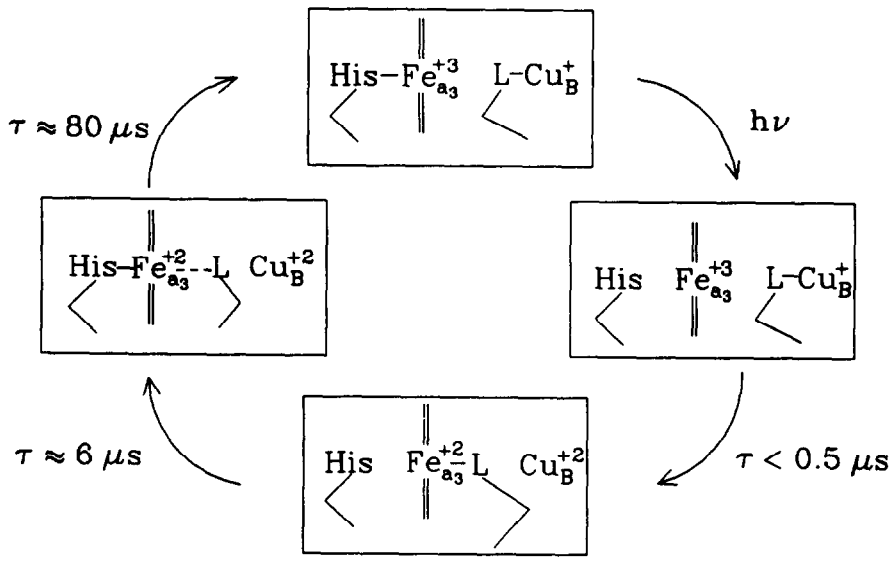

Fig. 4. A schematic diagram that models the experimental observations. See text for explanation.

cients of the reduced and oxidized cytochrome a_3 may differ from those of the transient states involved in the reactions observed in this study.

The ratio of the absorbance changes at 445 nm and at 605 nm, $\Delta A^{445}/\Delta A^{605}$ was ~ 10. This ratio is not consistent with that of reduced minus oxidized cytochrome a_3 (ratio is ~ 20) [23]. As indicated above, the reason may be that the extinction coefficients at 445 nm and 605 nm given in [23] may deviate from those of the transient states of the reaction observed here. For example, a transient alteration of the extinction coefficient at 605 nm due to structural changes in fully reduced COX has been observed previously [24].

According to the model shown in Fig. 4 the exchange of the proximal histidine for L does not give rise to absorbance changes; the observed absorbance changes are mainly due to electron transfer. Consequently, the ligand field from the histidine and from L must be similar. A potential candidate for L is therefore His-419 [25] of helix X (see [26]), which presumably is able to bind to either cytochrome a_3 or Cu_B.

According to one model for the mechanism of electron transfer in proteins the rate is greatly affected by the intervening medium; a through-space transfer is much slower than a through-bond transfer [27]. Consequently, a ligand which can bind to either cytochrome a_3 or Cu_B may constitute a unit which controls rates of electron transfer to these centres, an obligatory feature of the proton pump (see [28]). In addition, the ligand may also be involved in the oxygen binding to reduced COX, as indicated in [29].

Our results show that there are flash-induced structural changes in the vicinity of cytochrome a_3 in the absence of CO and O_2. These changes may occur also in other light-induced studies of COX and determine the kinetics of the reactions studied.

Acknowledgements: This study has been supported by grants from the Swedish Natural Science Research Council (K-KU 8897-304 to BP), the Erna and Victor Hasselblad Foundation, and the Knut and Alice Wallenberg Foundation. We are indebted to Professors Bo G. Malmström and Tore Vänngård for their support and helpful criticism. We have had very helpful discussions with Dr. Thomas Nilsson.

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