# ELECTRON REDISTRIBUTION IN CYTOCHROME *c* OXIDASE DURING FREEZING UNDER TURNOVER CONDITIONS

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## 1. Introduction

Studies on the functional involvement of the 4 redox centers (cytochromes *a* and $a_3$, $Cu_A$ and $Cu_B$) in the catalytic unit of cytochrome *c* oxidase (ferrocytochrome *c*:oxygen oxidoreductase, EC 1.9.3.1) have been dominated by the application of optical and EPR spectroscopy (review [1]). It has been assumed, to a large extent, that parallel experiments with the two techniques reflect the same states of the enzyme, despite the fact that different temperatures and protein concentrations have generally been used. This assumption has, however, been experimentally tested in a few cases only, for example, by the recording of optical spectra at the same temperature as used for the EPR measurements (see e.g., [2,3]).

In attempts to determine the spectroscopic states of the enzyme metal centers during turnover conditions, we observed apparent discrepancies between optical and EPR results. Thus, we found large decreases in the EPR signals (recorded around 20 K) from cytochrome *a* and $Cu_A^{2+}$ at times when there were no optical changes seen at room temperature. Comparisons with EPR spectra of samples prepared by a rapid-freeze technique and with optical data obtained at 77 K demonstrate that an intramolecular electron redistribution occurs during slow freezing which accounts for the experimental anomaly.

## 2. Materials and methods

### 2.1. *Enzyme*

Cytochrome oxidase was prepared from beef heart mitochondria by a slight modification of the method in [4]. The concentration of cytochrome oxidase was calculated from $\epsilon^{605}_{red-ox} = 24.0\ mM^{-1} \cdot cm^{-1}$ [5] and for cytochrome *c* from $\epsilon^{550}_{red-ox} = 21.1\ mM^{-1} \cdot cm^{-1}$ [6]. All experiments were made in 50 mM Hepes buffer (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) with 0.5% Tween 80, at pH 7.4. The activity of the cytochrome oxidase was 28 $s^{-1}$ measured in Hepes buffer as in [7].

### 2.2. *Optical and EPR measurements*

Optical spectra at 16°C were recorded in a Beckman Acta MIV spectrophotometer connected to a Hewlett Packard 2D-2AM *X–Y* recorder. Spectra at room temperature and 77 K (fig.3) were obtained with a Johnson Research Foundation DBS-2 dual wavelength spectrophotometer with the samples in quartz EPR tubes. EPR spectra at 77 K were recorded on a Varian E-3 spectrometer and at 18 K on a Varian E-9 spectrometer. Integrations of the signals were made as in [8].

### 2.3. *Turnover experiments*

A solution of 127 μM cytochrome oxidase was equilibrated with air at +16°C, and the spectrum of the oxidized enzyme was recorded in a 1 mm-pathway quartz cuvette. Then 2 μM cytochrome *c*, 7 mM sodium ascorbate and 5 μM TMPD (*N,N,N',N'*-tetramethyl-*p*-phenylenediaminedihydrochloride) were added to aerobic solution to initiate the catalytic reaction (final concentrations given). The concentrations were chosen in order to give an appropriately slow reaction rate. The solution was immediately transferred to an injection syringe for convenient sampling. Continuous repetitive scans from 700–400 nm were carried out on a sample drawn from the injection syringe in a Beckman Acta MIV spectrophotometer until almost complete reduction. In a separate

experiment, the 830 nm band was scanned in a similar way. Simultaneously, at short intervals, EPR samples were collected from the syringe directly into 3 mm (i.d.) quartz EPR tubes and frozen in ~2 s [9] in isopentane at −130°C. The samples were then transferred to liquid nitrogen.

### 2.4. *Rapid freeze*

Rapid-freeze experiments were performed as in [10] with an Update Instruments apparatus controlled by a Nova 3 mini-computer. The isopentane temperature was kept at −130°C with nitrogen gas bubbling through the cold isopentane bath, in order to keep the oxygen concentration low, thus minimizing oxidation of the enzyme during freezing [11]. The quenching time was estimated to be ~10 ms.

## 3. Results and discussion

The optical recordings during a turnover experiment are shown in fig.1. After addition of reductant to the aerobic cytochrome oxidase solution, hardly any optical changes can be seen over 400–900 nm until the oxygen has been consumed. Reduction of cytochrome oxidase is characterized by increases in the 605 nm and 445 nm bands and decrease in the 418 nm band, and such changes can be observed ~400 s after the addition of reductant. Fig.2A shows the time course of the optical changes at 605 nm and 418 nm replotted from fig.1. The 830 nm bands also decreased after a similar lag phase (not shown in fig.2A). Complete reduction of the enzyme was achieved in ~1800 s. Fig.2B gives the EPR data covering the same time interval as the optical results in fig.2A). There is an apparent discrepancy between the 2 sets of data. The EPR curve shows a rapid decrease

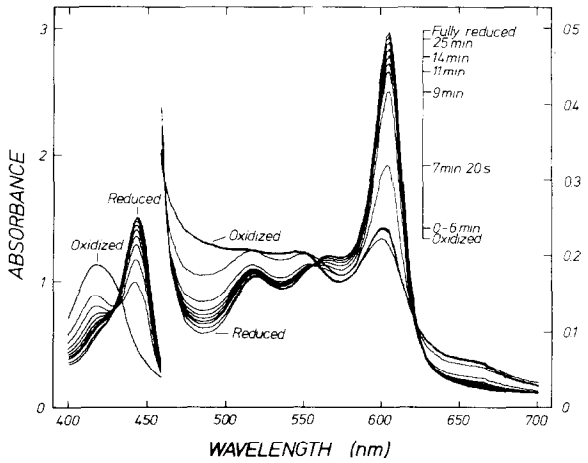


Fig.1. Time course of the optical changes of cytochrome oxidase from fully oxidized to fully reduced. A 127 μM cytochrome oxidase solution was flushed with air at 16°C and the reduction was initiated by adding 7 mM sodium ascorbate, 5 μM TMPD and 2 μM cytochrome *c*. All figures are final concentrations. When the sample was almost completely reduced, a few grains of dithionite were added to obtain a fully reduced spectrum. The absorbance scale is changed at 460 nm with a 6-fold increase in gain from 460–700 nm.

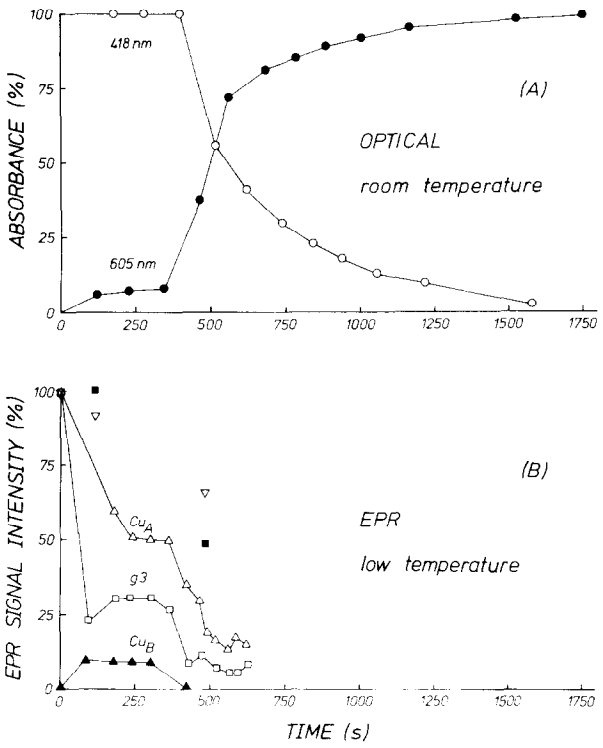


Fig.2. Time course of optical and EPR changes during the reduction of cytochrome oxidase. (A) Absorbance changes at 605 nm (●) and 408 (○), replotted from fig.1. (B) EPR signal intensities during reduction. $Cu_A$ (△), $Cu_B$ (▲), *g*3 (□), samples manually frozen in cold isopentane; $Cu_A$ (▽), *g*3 (■), samples from rapid-freeze experiments described in fig.4. The copper signals were recorded at 77 K; microwave frequency, 9.2 GHz; microwave power, 2 mW; modulation amplitude, 3.2 mT. The $Fe^{3+}$ *g*3 signal was recorded at 18 K; microwave frequency, 9.25 GHz; microwave power, 10.5 mW; modulation amplitude, 3.2 mT. The absorbance changes in (A) and the EPR intensity changes in (B) are related to the initial value of each band or signal in the resting enzyme. The $Cu_B$ signal, however, is in percent of the initial $Cu_A$ signal. Reductants were added at 0 s in both (A) and (B).

in intensity for both the $Cu_A^{2+}$ ($g2$) and the cytochrome $a^{3+}$ ($g3$) signals without any lag phase. A new $Cu^{2+}$ signal, ascribed in [12] to $Cu_B^{2+}$ in molecules having cytochrome $a_3$ reduced (lines over 0.26–0.29 T in fig.4), also appears, and it remains stable as long as oxygen is still present in the reaction mixture.

The apparent inconsistency between the optical and EPR recordings raised the question whether there existed EPR-detectable species during reduction that could not be seen optically. A more probable explanation for the observations would, however, be internal electron transfers by the difference in recording temperatures. To ascertain if the freezing of the EPR samples could affect the electron distribution, optical measurements at 77 K were performed. Optical spectra from cytochrome oxidase during turnover recorded both at room temperature and after freezing to 77 K are shown in fig.3. The room temperature spectrum (A) clearly represents an oxidized sample after addition of reductant (cf. fig.1), while after freezing in cold isopentane the same sample has been partially reduced (fig.3B). The sample was frozen ~200 s after the addition of reductant, i.e., in the middle of the lag phase (cf. fig.2A). For comparison, fig.3C shows the spectrum of the fully reduced sample. These effects of freezing the enzyme during turnover, were further examined by the rapid-freeze experiments illustrated in fig.4. The rapid-freeze EPR spectrum displays no $Cu_B^{2+}$ signal, the same $g3$ intensity as the oxidized enzyme and only a minor $g6$ signal. The sample quickly frozen by hand in cold isopentane at −130°C ('slow freeze' in fig.4), on the other hand, shows a decrease in intensity of the $g3$ signal, a much bigger nearly axial $g6$ signal and the $Cu_B^{2+}$ signal with $g_z = 2.27$ (lines over 0.26–0.29 T), features that are consistent with fig.2B (see inserted

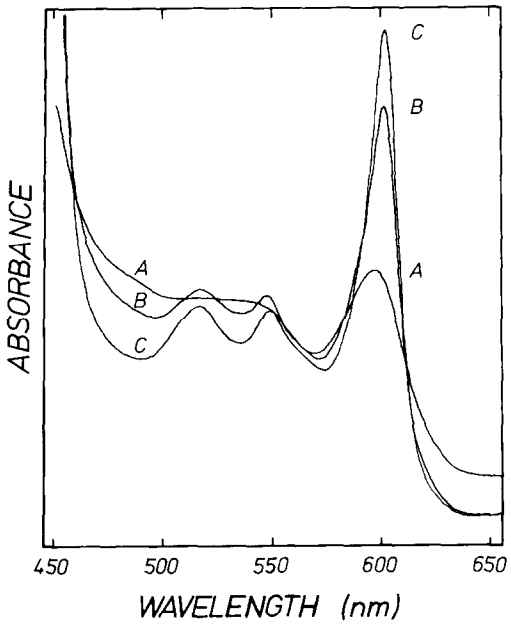


Fig.3. Optical spectra of cytochrome oxidase at room temperature and 77 K. (A) Cytochrome oxidase sample with reductant added. The spectrum was recorded at room temperature immediately after addition of reductant. (B) Optical spectrum at 77 K of the same sample as in (A), manually frozen after 200 s in cold isopentane during turnover. (C) Same sample as in (B) but thawed and reduced with a few grains of dithionite. The concentration of cytochrome oxidase was 50 μM, concentrations of ascorbate, TMPD and cytochrome *c* were the same as in fig.1. Since all spectra were recorded with the sample in EPR tubes, the absorbance scale is in arbitrary units.

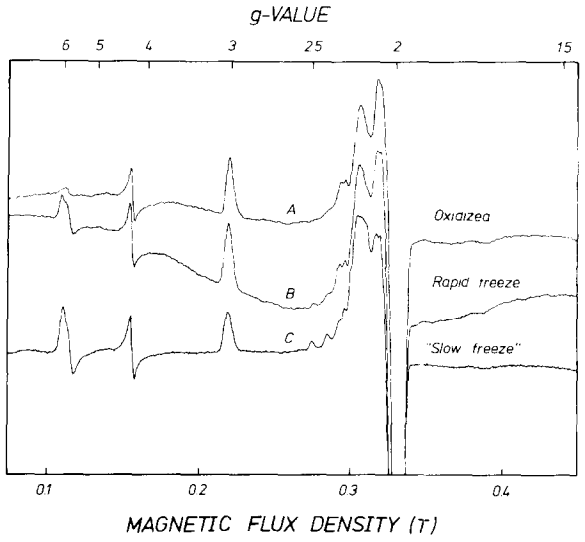


Fig.4. EPR spectra of cytochrome oxidase during turnover. (A) Oxidized reference sample (rapid freeze). (B) After recording spectrum (A), reductant was added to the enzyme solution which was then quickly transferred to the drive syringe of the rapid-freeze apparatus. A sample was then immediately frozen in the cold isopentane. Quenching time was ~10 ms. (C) Cytochrome oxidase sample, similar to (B) but frozen manually in cold isopentane (slow freeze). The samples in (B,C) were frozen during the lag phase of the reduction (after 105 s and 25 s, respectively; see fig.2). Enzyme concentration was 238 μM (A), 227 μM (B) and 214 μM (C). The concentrations of reductant were the same as in fig.1 for B,C. Microwave frequency, 9.25 GHz; microwave power, 2 mW; modulation amplitude, 2 mT; temperature, 18 K (A,B) and 20 K; the same gain was used in A–C.

intensities of the $g2$ and $g3$ rapid-freeze EPR signals for comparison).

There are two possible extrinsic electron sources only present in our system, namely ascorbate and water/oxygen. It is not likely that a decrease in temperature (i.e., freezing) should lead to an increase in the rate of electron transfer from the ascorbate–TMPD–cytochrome *c* system. A backflow of electrons from water/oxygen to cytochrome oxidase is thermodynamically unlikely and is, in addition, ruled out by the fact that, with reasonable estimation of freezing time and oxygen concentration in the solution, $\geqslant 80\%$ of the electron-accepting sites in the cytochrome oxidase molecule should still be oxidized.

As extrinsic donors seem ruled out, the electrons presumably come from reduced forms of cytochrome $a_3$ and $Cu_B$. A major difficulty with this interpretation is the small absorbance increase at 605 nm, indicating that cytochrome $a_3$ is essentially oxidized, and the very low intensity of the $g6$ signal, suggesting that few molecules with oxidized cytochrome $a_3$ has $Cu_B$ reduced. It should be noted, however, that mixed-valence-state cytochrome oxidase ($a^{3+}Cu_A^{2+}Cu_B^{1+}a_3^{2+}$) has an absorbance at 605 nm much closer to that of the oxidized than the reduced protein [13].

A possible origin of the reducing electrons is a radical observed in our experiments. A complication is, however, that this radical is variable. The radical does not arise from ascorbate as its EPR signal is also formed with other reductants (NADH + phenazine methosulfate). The possible relation of this signal, and of the $Cu_B^{2+}$ signal observable during turnover, to catalytically active species is now subject to further investigations in this laboratory. Our finding that freezing can effect electron redistribution in partially reduced cytochrome oxidase indicates that comparisons of results obtained at different temperatures must be made with caution with this as well as with other electron-transfer proteins. The rapid-freeze technique may be a means of avoiding this complication in studies involving partially reduced proteins, for example redox titrations or turnover experiments.

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