EPR EVIDENCE FOR AN ACTIVE FORM OF CYTOCHROME *c* OXIDASE DIFFERENT FROM THE RESTING ENZYME

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1. Introduction

As cytochrome *c* oxidase (cytochrome *c*: O_2 oxidoreductase, EC 1.9.3.1) is considered to contain two atoms of heme iron and two of copper in its functional unit [1,2], the EPR technique has played a prominent role in studies of the structure and function of this enzyme (see [3,4]). While in the resting oxidase EPR signals essentially from one low-spin heme Fe^{3+} and one Cu^{2+} only are observed, at least eight additional signals have been detected in various redox states of the enzyme [3]. Interactions between the different electron-accepting sites may, of course, lead to a large number of signals, but it is believed [3] that at least some of them arise from heterogeneities in the solubilized oxidase, such as differences in the degree of aggregation in solution. In view of this, attempts have been made to distinguish signals that reflect mechanistically important states of the enzyme from those that have no catalytic relevance. One criterion has been the rate of formation of signals appearing on reduction in relation to the rate of turnover in the catalytic reaction. On this basis the catalytic significance of one of the rhombic high-spin Fe^{3+} signals observed during anaerobic reduction has been questioned [4].

With the related oxidase laccase, it has been shown that reductive steps which appear to be too slow to be consistent with the turnover rate, become faster in molecules that have undergone at least one catalytic cycle [5]. The reason for this is an equilibrium in the resting enzyme between an active and an inactive enzyme form, the catalytic reaction displacing the equilibrium towards the active conformation. Recently, Antonini et al. [6] have demonstrated a similar situation with cytochrome oxidase, in which the so called 'pulsed' enzyme, i.e., reduced oxidase which has been reoxidized with a pulse of O_2, was shown to react more rapidly with ferrocytochrome *c* than the resting enzyme. It became, therefore, of interest to compare the rate of appearance of EPR signals in the resting and in the 'pulsed' oxidase. In this study we show that the earlier mentioned, slowly appearing high-spin Fe^{3+} signal forms rapidly when fully reduced oxidase is mixed simultaneously with oxygen and ferrocytochrome *c*. It is also demonstrated that the 'pulsed' enzyme has a different absorption spectrum in the visible region.

2. Materials and methods

Cytochrome oxidase was prepared from beef heart mitochondria essentially according to the method of van Buuren [7]. In order to remove the salt, gel-filtration on a Sephadex G-25 equilibrated with 0.1 M sodium phosphate buffer, pH 7.4, containing 0.5% Tween-80 or dialysis against the same buffer during 3 h with 3 changes was introduced as a last step. The activity was measured according to Smith [8] and was in this medium found to be about 40–50 s^{-1}. The absorbance ratio for the reduced enzyme at 444 nm and 420 nm was larger than 2.3 and the absorbance ratio for the oxidized form at 280 nm and 420 nm was less than 2.9 indicating that the preparations used contained very little nonreducible heme *a* and were without contaminating cytochromes. EPR measurements showed that extraneous copper amounted to less than 10% of the total EPR detectable copper. Horse

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heart cytochrome *c*, Type VI, from Sigma Chemical Co. was further purified by ion-exchange chromatography on Amberlite CG-50 [9]. The reduced form was obtained by addition of a small amount of sodium ascorbate followed by gel-filtration on Sephadex G-25 for removal of excess reductant. The eluted protein was directly made anaerobic by repeated pumping and flushing with oxygen-free nitrogen gas. Anaerobic titrations with ferricyanide confirmed the absence of ascorbate in the reduced cytochrome *c* solution. The concentrations of the cytochromes were determined from the difference in absorbance between the reduced and oxidized forms of the proteins, using an extinction coefficient of 24 $mM^{-1}\,cm^{-1}$ at 605 nm for cytochrome oxidase and 21 $mM^{-1}\,cm^{-1}$ at 550 nm for cytochrome *c*. Chemicals were from Merck Company or British Drug Houses Company and were all of analytical reagent grade.

The anaerobic system, the stopped-flow apparatus and the rapid-freeze system have been described elsewhere [10–12]. EPR spectra were recorded at 9 GHz with a Varian E-9 spectrometer. The temperature was kept at 12°K by a helium gas flow system [13]. Integrations of EPR spectra were made according to Aasa and Vänngård [14].

3. Results

Figure 1 shows the EPR results in the *g* 6 and *g* 3 regions of experiments in which cytochrome oxidase was reduced with ferrocytochrome *c* during different conditions as studied by the rapid-freezing technique. Starting with the resting, oxidized enzyme only one high-spin ferric heme signal is obtained in the *g* 6 region in the rapid phase with *g*-values 6.10 and 5.84 (fig.1 B). The same type of signal is obtained both in the presence and absence of oxygen. Figure 1 C shows the result obtained after 15 min of anaerobic incubation with one equivalent of ferrocytochrome *c*. The cytochrome oxidase low-spin heme is about 50% reduced as is also the case in fig.1 B, but the former high-spin signal is now replaced by essentially two new components with *g*-values 6.42 and 5.38 for the rhombic signal and 5.99 and 5.79 for the axial signal. If the partially reduced enzyme so formed is allowed to react with additional equivalents of ferrocytochrome *c*, the rhombic signal is decreased within 40 ms at 0°C (fig.1 D). Duplicate experiments showed more than 30%

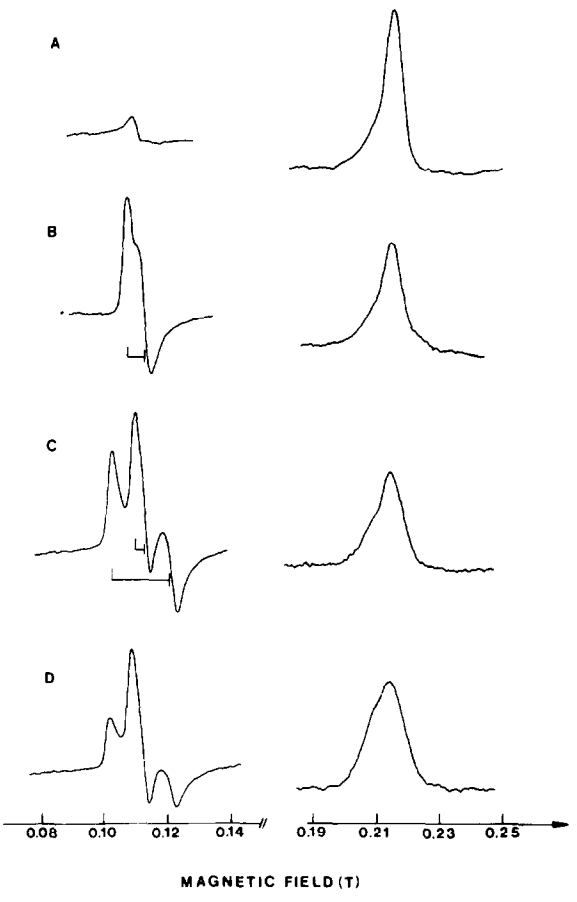

Fig.1. EPR spectra obtained in the *g* 6 and *g* 3 regions after rapid-freeze experiments with cytochrome oxidase. The reactions were carried out in 0.1 M sodium phosphate buffer containing 0.5% Tween-80. The enzyme concentration after mixing was 80 μM and the quenching time was 40 ms. The partially reduced enzyme was prepared by incubating 160 μM cytochrome oxidase anaerobically in a rapid-freeze syringe with one equivalent of ferrocytochrome *c* for 15 min at 0°C. The figure shows the results after mixing of (A) oxidized enzyme and one equivalent of ferricytochrome *c* in the presence of oxygen at 20°C, (B) oxidized enzyme and one equivalent of ferrocytochrome *c* at the same conditions, (C) partially reduced enzyme and anaerobic buffer at 0°C and (D) partially reduced enzyme and 1.5 equivalents of ferrocytochrome *c* in anaerobic buffer at 0°C. The *g*-values are in (B) 6.10 and 5.84 and in (C) 6.42, 5.38 and 5.99, 5.79 for the rhombic and axial components, respectively. The *g*-values of the different high-spin components are shown by the stick spectra, in which the lines above and across the base line indicate 'absorption' and 'derivative' type of lines, respectively. The spectra were recorded at microwave frequency, 9.13 GHz, microwave power, 2 mW, modulation amplitude, 2 mT and temperature, 15°K.

reduction of this signal. The g 3 region shows further reduction of cytochrome oxidase low-spin heme and the appearance of a broad signal, due to the formation of ferricytochrome *c*. The widths of these signals are 6 mT and 14 mT, respectively.

The same high-spin signals as seen in fig.1 C can also be formed within 40 ms by allowing the fully reduced enzyme to react with ferrocytochrome *c* and a stoichiometric amount of oxygen (fig.2 B). The total intensity of the high-spin signals corresponds to about 0.15 heme. With excess of oxygen, however, only small g 6 signals are obtained (fig.2 C).

Similar experiments were also performed at room temperature with the stopped-flow technique. From fig.3 it is evident that the product formed 10 ms after the reaction with reduced enzyme and oxygen is spectrally different from the resting enzyme. The Soret peak is shifted from 419 nm to 423 nm and the α-peak from 598 nm to 601 nm. In the Soret region there is a further redshift within the first 30 s towards the spectrum of the classical 'oxygenated' enzyme. The same result is also obtained with the partially reduced enzyme (not shown). Figure 4 shows that the partially reduced enzyme reacts rapidly with ferrocytochrome *c* in the presence of oxygen. Thus one equivalent of ferrocytochrome *c* is oxidized within 300 ms as seen from the 550 nm band. At 605 nm slow and complex changes occur after the rapid reactions with oxygen and ferrocytochrome *c*, and the final slow phase has a half-time of about 20 min.

4. Discussion

While EPR studies on the resting enzyme show essentially only low-spin heme iron (fig.1 A), various high-spin forms appear during reduction experiments with the enzyme. In the presence of oxygen the high-spin heme signal in fig.1 B is the only one seen during the whole course of oxidation of the reductant ferrocytochrome *c*. The intensity of this signal corresponds to less than 0.1 heme [4] and its origin is still obscure. It is formed rapidly, but its disappearance even in the presence of oxygen is fairly slow, usually taking a few

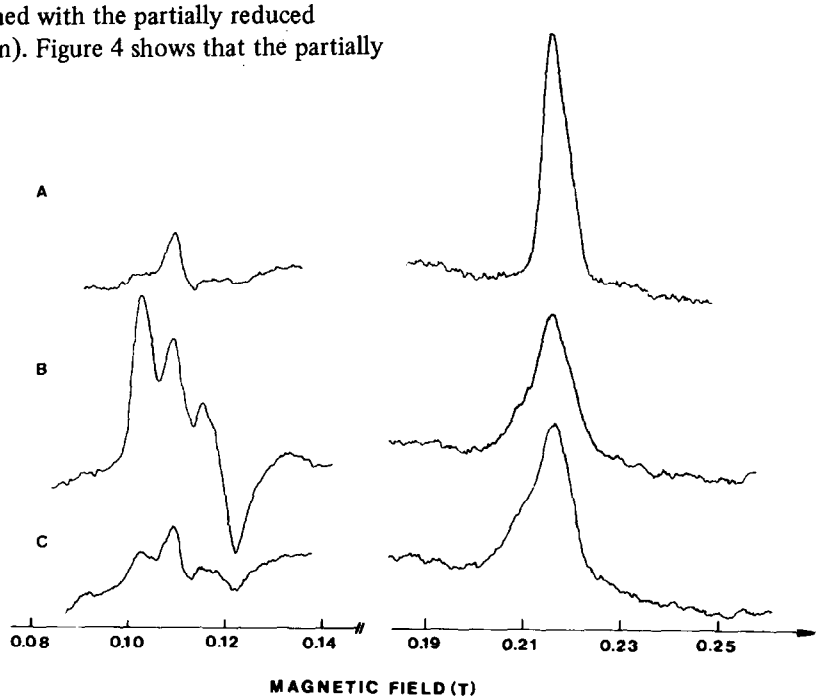

Fig.2. EPR spectra obtained in the g 6 and g 3 regions after rapid-freeze experiments with reduced cytochrome oxidase. The enzyme concentration was 100 μM after mixing. All signals are recorded at the same gain within their respective regions. Other conditions as in fig.1. The reduced form was obtained after incubation of the oxidized enzyme with 12 μM cytochrome *c* and 20 mM ascorbate for 30 min at 20°C. The content was thereafter mixed with (A) buffer containing 230 μM oxygen, (B) 1.6 equivalents of ferrocytochrome *c* in buffer containing 200 μM oxygen and (C) same as (B) but with 1.2 mM oxygen.

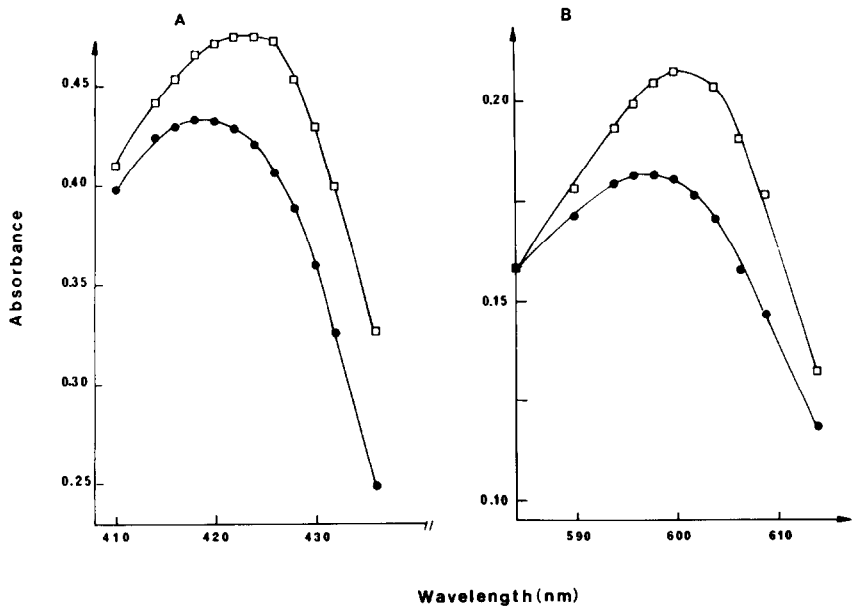

Fig.3. Optical absorbance spectra obtained in the 400 nm and 600 nm regions from stopped-flow experiments with cytochrome oxidase. The oxidized enzyme solution contained 0.2 μM ferricytochrome *c* and the reduced form was obtained by incubating this solution in the stopped-flow syringe with 15 mM ascorbate for 2.5 h. The temperature was 20°C and the medium was the same as in fig.1.(A) shows the spectrum in the 400 nm region 10 ms after mixing of oxidized (●-●-●-●) and reduced (□-□-□-□) enzyme with buffer containing 230 μM oxygen. The enzyme concentration was 1.5 μM after mixing. In (B) is shown the spectrum obtained in the 600 nm region of the same experiments, but in this case the enzyme concentration was 5 μM after mixing. The pathlength was 2.0 cm and the band width was 1.4 nm in the 400 nm region and 2.8 nm in the 600 nm region.

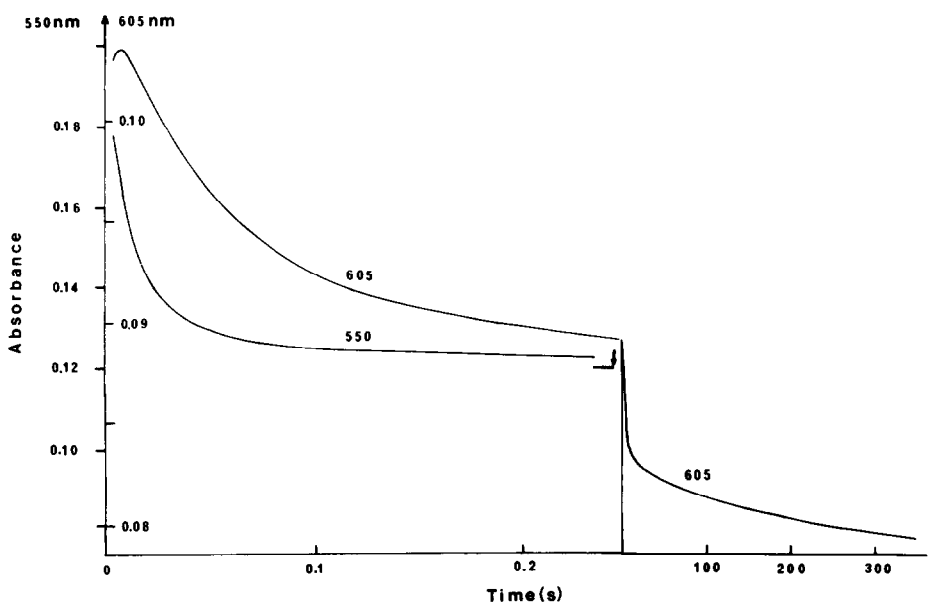

Fig.4

seconds when reduction has been performed with approximately stoichiometric amounts of ferrocytochrome *c*. In anaerobic reduction experiments, this signal is slowly replaced by the high-spin heme components shown in fig.1 C, which start to form within one second. The intensity finally increases to about 0.2 heme, with the rhombic component responsible for the major part, when one equivalent of ferrocytochrome *c* has been added [15]. Though slowly formed, the rhombic signal responds rapidly to both ferrocytochrome *c* (fig.1 D) and oxygen (fig.2 C and [4]) and it can also be formed rapidly in reduction experiments (fig.2 B). It has earlier been shown that the rhombic signal is obtained in experiments where the reduced enzyme is oxidized by ferricytochrome *c* anaerobically [16]. It might thus be argued that the rhombic signal in fig.2 B appears as a result of an oxidation reaction between reduced enzyme and ferricytochrome *c*. A kinetic analysis shows, however, that the simultaneous levels of oxidized cytochrome *c* formed and remaining reduced cytochrome oxidase molecules will never be high enough to produce more than maximally 10% of the observed *g* 6 signal. Thus, on reoxidation, a species is formed different from the resting enzyme in its reaction with ferrocytochrome *c*. The rhombic *g* 6 signal produced on reduction may well be significant in the catalytic reaction.

Our optical studies show that the species formed rapidly in the reaction between fully reduced enzyme and oxygen also differs from the classical 'oxygenated' compound. This has an absorption maximum at 426–428 nm (see [17,18]) whereas the rapidly formed product has a maximum around 423 nm (fig.3 A). The two forms have the same absorption maximum (601 nm) in the α-region (fig.3 B and [17]) different from that of the resting enzyme (598 nm).

There are three different species with the same EPR spectrum but with distinctly different optical properties, the resting protein, the oxygenated compound and the species observed here on rapid oxidation. Thus, in this case EPR does not distinguish between different molecular forms. It might be argued that the low temperatures required for EPR studies could shift equilibria favoring one form only. However, optical spectroscopy at liquid nitrogen temperature shows that no shift in the position of the α-band occurs on freezing at least for the resting and rapidly formed compound.

Reoxidation of the fully and partially reduced enzyme leads to the rapid formation of compounds with the same EPR and optical spectra (fig.3 and [4]) both reacting rapidly with ferrocytochrome *c* (fig.4 and [6]). One would expect that different species should be formed, in particular since the intermolecular transfer is relatively slow [17]. In fact one cannot exclude the existence of an oxygen intermediate relatively stable even at room temperature. Thus, for the tree laccase, under certain conditions an intermediate is stable for about 10–15 s [19].

The results presented in this work show that cytochrome oxidase can exist in an active form different from the resting enzyme, and it is likely that this form is the same as that recently described by Antonini et al. [6]. Under their conditions the 'pulsed' enzyme has a higher turnover number than the resting enzyme. This may be a result of a different conformation with different electron distribution or different electronic properties. There may be a close analogy to other oxidases, as it has been shown [5] that the active form of laccase from *Rhus vernicifera* is not obtained before the enzyme has undergone one turnover.

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Fig.4. Stopped-flow experiments with partially reduced cytochrome oxidase and ferrocytochrome *c* in the presence of oxygen. The experiments were carried out in the same medium as in fig.1 and the temperature was 25°C. The partially reduced enzyme was prepared by incubating 24 μM oxidized enzyme anaerobically with 26 μM ferrocytochrome *c* for 15 min at 23°C whereafter the content was transferred anaerobically into one the stopped-flow syringes. This solution was then mixed with 24 μM ferrocytochrome *c* so that the final concentrations were 12 μM and 25 μM for the oxidase and cytochrome *c*, respectively. The arrow shows the final absorbance value at 550 nm. At 605 nm, equilibration was reached after about 1 h with a final absorbance value of 0.058. The time scale contracts at the vertical line. The path length was 2.7 mm and the band width was 2.8 mm.

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