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# KINETIC STUDIES ON OXIDIZED AND PARTIALLY REDUCED CYTOCHROME *c* OXIDASE

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## Summary

1. Kinetic studies have been performed with beef-heart cytochrome *c* oxidase, with the enzyme either in its oxidized, resting state or pretreated anaerobically with different amounts of reduced cytochrome *c*. The techniques used for the study have been stopped-flow spectrophotometry and electron paramagnetic resonance (EPR) spectroscopy.

2. The results show that the one-electron equivalent-reduced enzyme rapidly oxidizes one further equivalent of aerobically or anaerobically added ferrocytochrome *c*, with a rate constant of $5 \cdot 10^6\ M^{-1} \cdot s^{-1}$.

3. When an excess of ferrocytochrome *c* in the presence of oxygen is added to the one-electron-reduced enzyme, the same turnover rate is obtained as in experiments with the resting enzyme.

4. The one-electron equivalent-reduced enzyme reacts with CO with a rate constant of $4 \cdot 10^4\ M^{-1} \cdot s^{-1}$ to yield approx. 35% of the CO compound as compared with the reaction between the fully reduced enzyme and CO.

5. It is shown that on reduction the enzyme is converted into an active form, but it is concluded that the enzyme does not have to be fully reduced before it is catalytically active.

## Introduction

Extensive studies on cytochrome oxidase (ferrocytochrome *c* : $O_2$ oxidoreductase, EC 1.9.3.1), the terminal component of the respiratory chain, have shown that this enzyme behaves in a complex way both in reduction experiments and in its reaction with oxygen [1—5]. The properties of the two heme components and two copper atoms, which are considered to reside in the smallest functional unit, have been studied with different approaches [2,6—8]. The partially reduced enzyme shows EPR behaviour quite distinct from that

of the resting enzyme, as manifested by the appearance of high-spin heme signals, which may amount to maximally 0.5 heme. Redox experiments have been interpreted to show the existence of two high-potential and two low-potential components. The partially reduced enzyme is, however, poorly characterized with regard to its kinetic properties. These are of interest as they may give information about changes in the accessibility of reductant and intramolecular electron transfer, as compared to the resting and fully reduced enzyme. This paper presents the results of kinetic studies on the partially reduced enzyme with ferrocytochrome *c*, oxygen and carbon monoxide.

## Materials and Methods

Preparation and properties of cytochrome oxidase and cytochrome *c* and the experimental techniques used in the rapid-kinetic studies were as described earlier [9]. For some experiments, the enzyme used was prepared by hydrophobic interaction chromatography [10]. Spectrophotometry at liquid nitrogen temperature was performed with a DBS-1 Johnson Research Foundation dual wavelength instrument. Chemicals were from Merck Co. or British Drug Houses Co. and were all of analytical reagent grade. Carbon monoxide came from Matheson Gas Co. In order to remove traces of oxygen, the gas was stored for at least 24 h in a container with alkaline dithionite solution.

Partially reduced cytochrome oxidase was obtained by incubating anaerobically various amounts of ferrocytochrome *c* with the enzyme for at least 20 min. One-electron- and two-electron-reduced enzyme refers to the equilibrium mixture obtained after adding one- and two-electron equivalents of ferrocytochrome *c*, respectively. The anaerobicity of the stopped-flow system was tested by mixing anaerobic solutions of partially reduced enzyme and phosphate buffer, and the absence of any absorbance changes at 445 nm was taken as evidence for a negligible oxygen content.

## Results

*Reactions with reduced cytochrome c*

Fig. 1, curves a and b, shows the amount of ferricytochrome *c* formed in anaerobic reduction experiments with partially reduced cytochrome oxidase as studied by the stopped-flow technique. It is evident that the one-electron-reduced enzyme rapidly oxidizes about one further equivalent of ferrocytochrome *c* while the two-electron-reduced enzyme only oxidizes a small amount of cytochrome $c^{2+}$. An apparent second-order rate constant of $5 \cdot 10^6\ M^{-1} \cdot s^{-1}$ for the rapid reaction between ferrocytochrome *c* and one-electron-reduced enzyme was estimated from experiments with lower concentrations of reactants. Plots of extinction changes at 550 nm versus those at 605 nm yielded straight lines with slopes 2.0—2.4, whereas similar plots from experiments with the resting enzyme gave values of 1.5—1.8.

Similar experiments with the one-electron-reduced enzyme were also performed with the rapid-freezing technique, and EPR spectroscopy at 12 K confirmed the results obtained at room temperature regarding the amounts of ferricytochrome *c* formed (for a qualitative description, see ref. 9, Figs. 1C

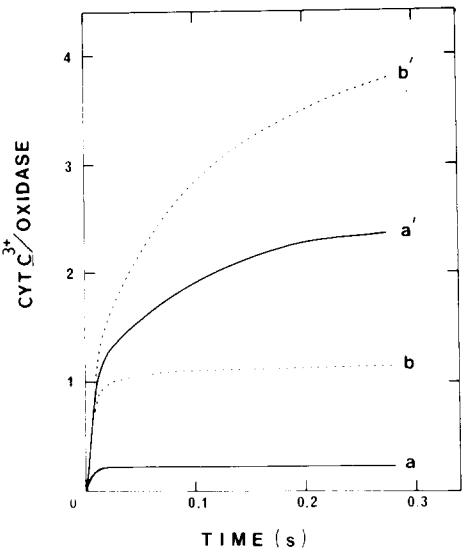


Fig. 1. Time course of the oxidation of ferrocytochrome *c* by partially reduced cytochrome oxidase. The reactions were followed in the stopped-flow apparatus at 550 nm (path length 2.7 mm, band width 1.6 nm) in 0.1 M sodium phosphate buffer, pH 7.4, containing 0.5% Tween 80. The temperature was 25°C. For preparation of partially reduced enzyme, see Materials and Methods. The solid lines show the reactions of 2.2 equivalents of ferrocytochrome *c* with 2.3-electron-equivalent reduced cytochrome oxidase. In a, the reactants were mixed anaerobically, while in a', the cytochrome *c* solution was aerobic. The enzyme concentration was 13 μM and the oxygen concentration was 115 μM after mixing. The dotted lines show the corresponding reactions of 4.6 equivalents of ferrocytochrome *c* with one-electron-equivalent reduced cytochrome oxidase. In this case the enzyme concentration was 8 μM.

and 1D). The peak positions of the enzyme in the α-band region were the same as those obtained at room temperature. Shape analysis of the *g*3 region showed that the cytochrome oxidase low-spin heme changed from 50% to more than 70% reduction (the presence of the relatively large amounts of cytochrome $c^{3+}$ prevented a better estimation), but the intensity of the copper signal did not appear to change.

*Oxidation experiments*

Fig. 2 shows absorbance changes at 420 and 428 nm in reoxidation experiments of one-electron equivalent-reduced enzyme with oxygen. After a rapid initial increase there is a very slow decrease of the absorbance at 428 nm which is not completed until after approx. 1 h. There is an accompanying slow increase at 420 nm, but the absorbance changes at the two wavelengths are not linearly correlated. The product obtained after the rapid reaction with oxygen was found to have an apparent extinction coefficient at 605 nm of about 28 $mM^{-1} \cdot cm^{-1}$. Experiments with 1.9 μM enzyme and 3 μM oxygen gave an apparent rate constant of $1 \cdot 10^8\ M^{-1} \cdot s^{-1}$ for the initial reaction with oxygen.

Reoxidation experiments performed with the rapid-freezing technique showed EPR results consistent with those earlier reported [11], i.e. the rhombic high-spin heme signal(s) disappeared and the maximal low-spin heme and copper signals appeared within 40 ms, and the axial high-spin heme signal

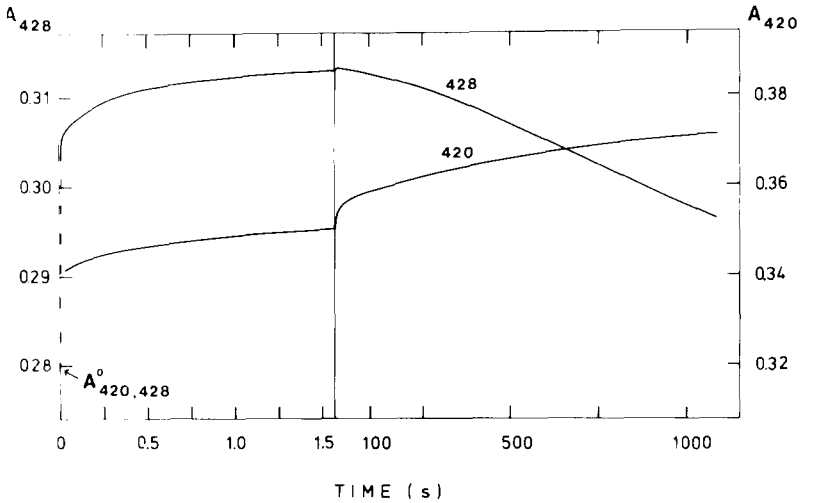


Fig. 2. Time course obtained at 420 and 428 nm on reoxidation of one-electron-equivalent reduced cytochrome oxidase with oxygen. The experiment was carried out in the stopped-flow apparatus. The same medium as in Fig. 1 was used. For preparation of the partially reduced enzyme, see Materials and Methods. The enzyme concentration was 5.8 μM and the oxygen concentration 115 μM after mixing. The time scale contracts at the vertical line. The path length was 2.7 mm and the band width 1.4 nm.

was about 80% reduced at the same time. Experiments with neither the partially nor the fully reduced enzyme yielded any EPR evidence of intermediate species such as found with laccase [12].

*Reactions with reduced cytochrome c in the presence of oxygen*

Fig. 1, curves a′ and b′, shows the results of reduction experiments with one- and two-electron equivalent-reduced enzyme in the presence of 115 μM oxygen. Similar experiments with the one-electron-reduced enzyme were also performed with the rapid-freezing technique. After a reaction time of 40 ms EPR studies showed a 30% reduction of the cytochrome oxidase low-spin heme and no significant reduction of the copper signal as compared to the oxidized enzyme and there was only a minor appearance of high-spin heme signals.

For comparison, similar experiments were also done with the resting enzyme, and Fig. 3 shows the absorbance changes at 428, 445 and 550 nm in an experiment with 6.2 equivalents of ferrocytochrome *c* added in the presence of 115 μM oxygen. The absorbance changes have been corrected for contributions from cytochrome *c*, using the values +4.4 and −8.8 $mM^{-1} \cdot cm^{-1}$ at 428 and 445 nm, respectively, for the differences in molar absorbance between the reduced and oxidized forms. Comparisons of the 550 nm trace with Fig. 1, trace b′, show that the rate of oxidation of ferrocytochrome *c* after the initial phase is about equal. Experiments performed with the resting and partially reduced enzyme, using 3.8 μM cytochrome oxidase and 9.7 μM cytochrome *c* in both cases, confirmed this finding. The absorbance at 428 nm in Fig. 3 can be accounted for as contributions from partially reduced and oxidized enzyme, where the proportion of partially reduced enzyme is determined from the absorbance at 445 nm at corresponding times. Thus, there is no indication of any 428 nm intermediate.

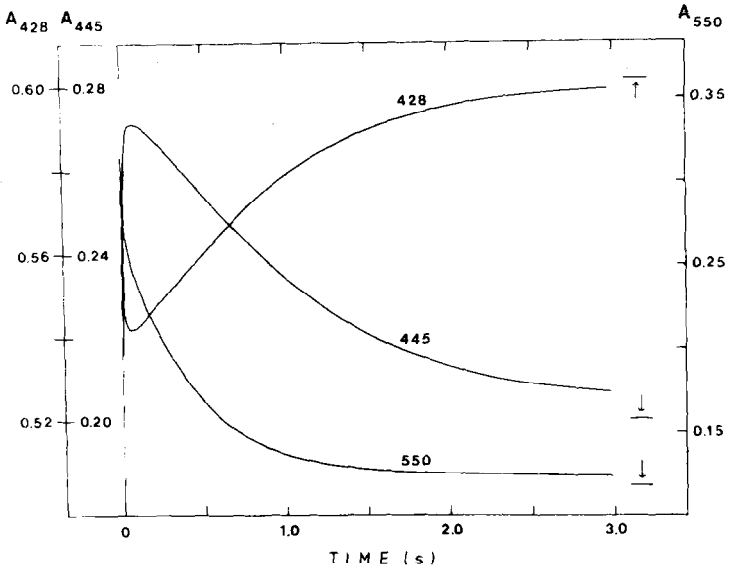


Fig. 3. Stopped-flow experiments with oxidized cytochrome oxidase and ferrocytochrome *c* in the presence of oxygen. The same medium as in Fig. 1 was used. Concentrations of reactants after mixing: cytochrome oxidase, 6.3 μM; cytochrome *c*, 39 μM; and oxygen, 115 μM. The arrows show final absorbance values at respective wavelengths. Changes due to cytochrome *c* have been corrected for at 428 and 445 nm.

The same type of experiments was also performed under true steady-state conditions and investigated by both the stopped-flow technique and by EPR. 0.2 μM enzyme was mixed with 20 μM ferrocytochrome *c* in the presence of 115 μM oxygen and the oxidation of reduced cytochrome *c* was monitored at 550 nm in the stopped-flow spectrophotometer. The rate of oxidation expressed as number of mol of ferrocytochrome *c* oxidized per mol of enzyme was 20 $s^{-1}$ under these conditions and there was no evidence of any lag in the beginning of the reaction (see Discussion). In another experiment, 5 μM enzyme was mixed with 200 μM ferrocytochrome *c* in the presence of 230 μM oxygen. The reaction was stopped after approx. 7 s by transfer of the tube into a cold (160 K) isopentane solution. EPR investigation at 5 K showed only the same high-spin signal as formed at early reaction times in reduction experiments with cytochrome *c* [9,13] and its intensity corresponded to less than 10% of the enzyme concentration.

*CO experiments*

Fig. 4 shows the absorbance changes at 445 nm of experiments in which cytochrome oxidase was reduced anaerobically with ferrocytochrome *c* (trace a) and where CO was added either simultaneously with the reductant (trace b) or 20 min afterwards (trace c). It is apparent that the kinetics of the CO reaction is quite different in traces b and c, as judged from the 445 nm change, but the final values are the same as expected when normalized to the enzyme concentration. Comparisons between the reaction of the fully reduced enzyme with CO showed that 32—40% of the maximal CO reaction was obtained with

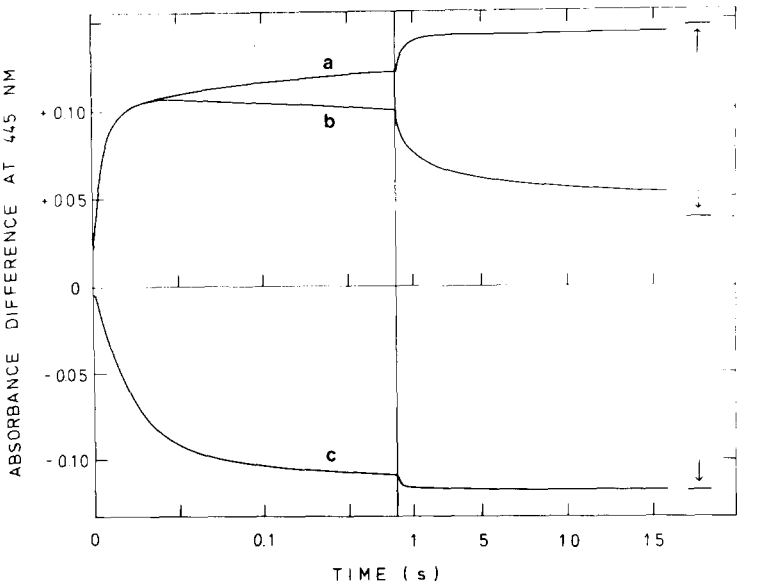


**Fig. 4. Anaerobic stopped-flow experiments with cytochrome oxidase and cytochrome *c* in the absence and presence of CO. The experiments were carried out in the same medium as in Fig. 1. For preparation of partially reduced enzyme, see Materials and Methods. Trace a shows the reaction between 15.5 μM oxidized cytochrome oxidase and 16 μM ferrocytochrome *c*. Trace b shows the same experiment but in this case CO was included in the cytochrome *c* solution. Trace c shows the reaction between one-electron-equivalent reduced cytochrome oxidase and CO. In both b and c, the CO concentration was 0.5 mM. The enzyme concentration was 16.3 μM. All values refer to the concentrations after mixing. The time scale contracts at the vertical line, and the arrows show the final absorbance values.**

the one-equivalent-reduced enzyme, assuming that the extinction coefficient for the CO compound was the same in the two experiments. The apparent rate constant of the CO reaction in Fig. 4c was estimated to $4 \cdot 10^4\ M^{-1} \cdot s^{-1}$.

## Discussion

From the experimental results it is obvious that the one-electron-reduced enzyme differs from the partially reduced enzyme formed after the initial reduction with cytochrome *c* in that it reacts rapidly with both ferrocytochrome *c* (Fig. 1), oxygen (Fig. 2) and carbon monoxide (Fig. 4). It is suggested that some of the enzyme molecules which possess these characteristics are catalytic intermediates, and in the following the properties of these molecules will be closer examined.

On mixing ferrocytochrome *c* with oxidized cytochrome oxidase one equivalent of ferricytochrome *c* is rapidly formed [14], a result which has been confirmed with the preparations used in this study. During anaerobic conditions rhombic high-spin heme is formed slowly [11], and Fig. 4b shows that the binding of CO also occurs at a low rate [15]. The one-electron-reduced enzyme, however, reacts rapidly with CO (Fig. 4c), and EPR spectroscopy reveals concomitant changes in the high-spin heme region [11]. It is difficult to estimate the distribution of electrons among the enzyme molecules in the one-electron-reduced system, as an analysis shows that the available redox equili-

brium data cannot be accounted for in terms of independent electron-accepting sites [16]. When interaction is included, however, no unique solution can be obtained [16].

In the reaction between one-electron-reduced enzyme and ferrocytochrome *c* a linear correlation is obtained for the absorbance changes at 550 and 605 nm, and the extinction coefficient calculated at 605 nm is of the same order as in reactions with the resting enzyme. This clearly indicates that a heme component in cytochrome oxidase is involved. Since the rate constant for the reaction between ferrocytochrome *c* and the one-electron-reduced cytochrome oxidase is the same as that obtained with the resting enzyme, it is suggested that the same acceptor is involved. The simplest explanation of the data would be that the primary acceptor in the one-electron-reduced enzyme is reoxidized and able to accept more reductant. EPR studies in the *g*3 region indicate that the low-spin heme of cytochrome oxidase is the primary acceptor of electrons from ferrocytochrome *c* [11]. The *g*3 signal is, however, only 50% developed in the one-electron-reduced enzyme, thus speaking against full reoxidation of this component. The data are therefore best explained by an increase of the rate of intramolecular electron transport from the primary acceptor to other sites in the enzyme, and this conclusion is irrespective of the distribution of electrons.

An important question is which enzyme molecules that react with $O_2$. From reoxidation experiments with the one-electron-reduced enzyme it is known that the *g*3 signal is fully recovered within 40 ms (see Results), the change in intensity corresponding to about 0.5 heme. There is also a large and rapid increase of the absorbance at 428 nm during the same time period (Fig. 2), indicating formation of oxygenated enzyme [17]. As EPR spectroscopy on the one-electron-reduced enzyme argues against a situation where 50% of the molecules contain two electrons, the implication is that some enzyme species containing only one electron also react with $O_2$. The absence of new paramagnetic signals on reoxidation is no conclusive evidence against this concept, since a narrow signal with low *g* value (cf. the $O_2$ intermediate in laccase [12]) could be hidden in the *g*-perpendicular region of the copper signal. A large fraction of the one-electron-reduced enzyme also reacts with ferrocytochrome *c*, and it is probable that some molecules can react with either cytochrome *c* or $O_2$. There is, in fact, experimental evidence for this, as at least some of the molecules which contain rhombic high-spin heme respond rapidly to both reagents [9,11].

Since the turnover rates are equal whether one starts with the partially reduced or with the resting enzyme (Fig. 1, traces a′ and b′, Fig. 3), it is probable that the same active enzyme species is involved. In line with the results obtained from reduction experiments in the presence of oxygen (Fig. 3), no lag was observed at 550 nm after the initial burst in experiments with large excess of substrate (100 : 1). One explanation is that the presence of oxygen leads to a rapid formation of catalytic intermediates, containing rhombic high-spin heme and that these molecules in turn communicate effectively with oxygen. The finding that only the nearly axial high-spin heme signal is observed in steady-state experiments is also consistent with this hypothesis.

The effect of oxygen on cytochrome oxidase is probably not the same as

with the laccases, with which oxygen has been shown to displace an equilibrium between inactive and active enzyme forms [18]. This conclusion is in contrast to a recent suggestion [19], that the resting enzyme has to be fully reduced before it can be converted to an active form, effectuated by a "pulse" of oxygen. It is shown in this paper that the two-electron-reduced enzyme only oxidizes a very small amount of ferrocytochrome *c* (Fig. 1, trace a), and it is therefore improbable that the fully reduced state ever is obtained during reactions with ferrocytochrome *c* in the presence of oxygen. The fact that already the partially reduced enzyme reacts rapidly with oxygen (Fig. 1, compare traces b and b′ and Fig. 2) also speaks against any formation of fully reduced enzyme molecules when oxygen is present.

It is important to emphasize that the experiments presented in this paper do not offer a unique interpretation. Thus, although it has been shown earlier, that the rhombic high-spin heme is formed rapidly under certain conditions [9] one cannot conclude with certainty that this species is formed in turnover experiments when starting with the resting enzyme, since the experiments presented in this paper do not take into consideration all steps in the catalytic mechanism. It is also possible that only a small fraction of the enzyme molecules are responsible for all the activity in the purified preparation, and that the species containing rhombic high-spin heme only has a negligible contribution to the turnover rate. Furthermore, it is worth noticing the difficulties in correlating rhombic high-spin heme signals with activities. Thus, although the mitochondrially bound enzyme is considerably more active than the purified preparation, the same high-spin heme signals are obtained in both cases when cytochrome oxidase is partially reduced [6]. An illustration of the complexities of this enzyme is the recent finding of a high-spin heme signal with a different shape which has been observed in reduction experiments with cytochrome oxidase, which has been incorporated in phospholipid vesicles, constituting a highly active system [20].

The essential information in the present paper is, however, that after reduction the approach to equilibrium leads to partially reduced forms that react rapidly with both ferrocytochrome *c* and oxygen, and the fully reduced state is therefore probably not a compulsory step for a conversion of the resting enzyme into active species.

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