THE REACTION BETWEEN OXIDIZED CYTOCHROME *c* AND REDUCED CYTOCHROME *c* OXIDASE

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1. Introduction

The elucidation of the reaction mechanism of isolated cytochrome *c* oxidase has been approached by methods involving equilibrium studies, steady-state and transient-state kinetics (see [1] and [2] for reviews). The reaction of the oxidase with its natural substrate, ferrocytochrome *c*, has for obvious reasons received a good deal of attention. The transient-state kinetics of this reaction, investigated in the presence and absence of oxygen, consist of several phases, the first of which is a second-order reaction, with a rate constant of 10^6–10^7 M^{-1} s^{-1} [3–7]. Direct measurement of the stoichiometry of the reaction, using the product, ferricytochrome *c*, as a probe, indicates that only one of the possible four electron acceptors of the oxidase, cytochrome *a*, is reduced in the burst phase [5,7]. The establishment of a quasi-equilibrium permits the calculation of an equilibrium constant for the redox reaction. This corresponds to an oxidation–reduction potential for cytochrome *a* of 285 mV, which is significantly different from the value found in potentiometric titrations. Together with other apparently conflicting observations concerning the properties of the prosthetic groups in cytochrome *c* oxidase (see [2]) this finding emphasizes the complexity of the enzyme. In an attempt to investigate further the oxidation–reduction sites of the enzyme, we report in this paper studies on the reaction between ferricytochrome *c* and reduced cytochrome *c* oxidase using stopped-flow and rapid-freeze e.p.r. methods. Our measurements strongly indicate that the quasi-equilibrium between ferricytochrome *c* and the reduced oxidase is different from that established with ferrocytochrome *c* and the oxidized oxidase with respect to stoichiometry and to midpoint potentials of groups involved. This is taken as evidence for site–site interaction within the oxidase molecule. Maximally two of the possible four sites in the enzyme are oxidized by ferricytochrome *c* in a transient phase. The oxidation is accompanied by some 50% decrease in the absorption peaks of the reduced enzyme at 605 and 445 nm and is characterized by the appearance of oxidized high-spin heme and copper in the e.p.r. spectra, while the low-spin signal seen in the fully oxidized oxidase is virtually absent.

2. Materials and methods

Cytochrome *c* oxidase was prepared from submitochondrial particles according to van Buuren [8]. The concentration of cytochrome oxidase is expressed in terms of a unit containing two hemes *a* and two copper ions. The maximal activity of this preparation in 0.1 M potassium phosphate 0.5%. Tween-80, pH 7.4 25°C, given by the turnover at excess cytochrome *c* in the polarographic assay is 120 s^{-1}. $A^{red}_{603}/A^{red}_{485}$ and $A^{red}_{444}/A^{red}_{424}$ were 4.4 and 2.4 respectively indicating an optically clear preparation with little non-reducible heme *a*. Cytochrome *c* (Sigma Type VI) was further purified by ion exchange chromatography [9]. The solution containing ferri-

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cytochrome *c* and the one containing cytochrome *c* oxidase was depleted for oxygen as previously described [10]. Reduction of cytochrome *c* oxidase was then obtained in the presence of a small concentration of cytochrome *c* by the addition of ascorbate. The stopped-flow apparatus, the rapid-freeze apparatus and methods for the evaluation of the stopped-flow traces have been described elsewhere [10,11]. E.p.r. spectra were recorded with Varian E-3 or E-9 spectrometers. For measurements below 77°K a helium gas flow system was used [12]. Integration of the e.p.r. signals were performed according to [13,14] using a copper standard for comparison. The experiments were carried out at 25°C in potassium phosphate buffer, pH 7.4, containing 0.5% Tween-80.

3. Results

In the anaerobic reaction between ferricytochrome *c* and reduced cytochrome *c* oxidase, electrons are rapidly transferred from the oxidase to cytochrome *c* as demonstrated in fig.1. The initial reduction of cytochrome *c* measured at 550 nm occurs simultaneously with a rapid oxidation of cytochrome *c* oxidase observed as a decrease in the absorbance at 605 nm. This phase was followed by a much slower reduction of cytochrome *c* and finally of the oxidase due to the presence of excess ascorbate in the oxidase solution. The second order constant for the initial reaction between ferricytochrome *c* and the oxidase (aa_3 unit) is 6×10^6 M^{-1} s^{-1} at 100 mM potassium phosphate. When the phosphate concentration was decreased to 60 mM, a two-fold increase in the rate constant was observed but the amount of cytochrome *c* reduced was not changed.

The amount of cytochrome *c* reduced by the oxidase in the initial burst phase of the reaction was determined by extrapolation of the slow phase as shown in fig.1. This amount will vary with the concentration of the cytochrome *c* oxidase, $[aa_3]$ and the initial concentration of ferricytochrome *c* $[c^{3+}]_o$.

The effect on the burst phase of varying $[c^{3+}]_o$ is shown in fig.2. The number of electrons per oxidase

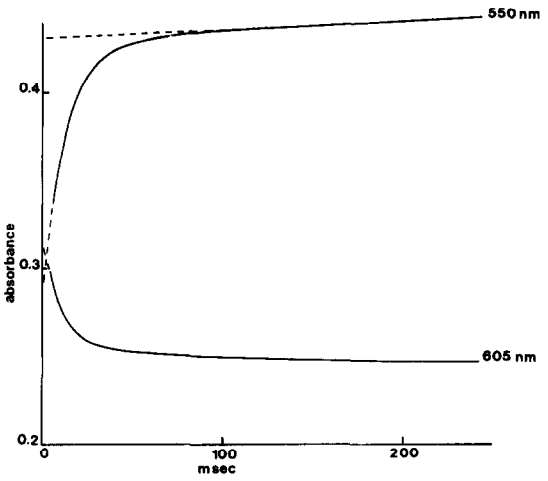


Fig.1. Time course of the anaerobic reaction between reduced oxidase and oxidized cytochrome *c*. The reaction was followed at 550 nm and 605 nm. The experiments were carried out in the stopped-flow apparatus (2 cm optical path) at 25°C in 0.1 M potassium phosphate, pH 7.4, containing 0.5% Tween-80. The concentration of cytochrome oxidase and cytochrome *c* after mixing was 3.1 μM and 6.5 μM respectively.

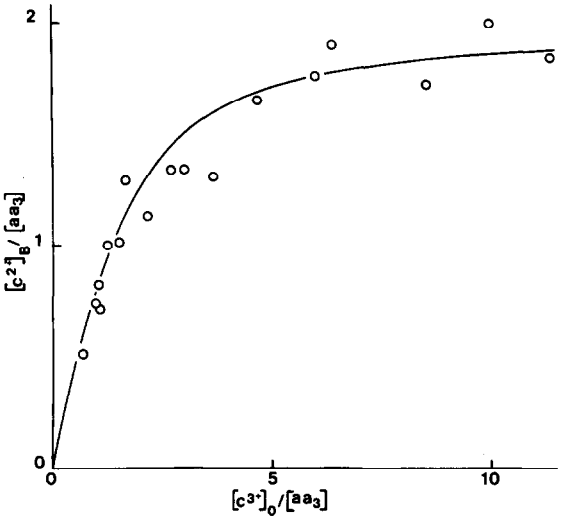


Fig.2. Equivalents of ferrocytochrome *c* $[c^{2+}]_B/[aa_3]$, formed during the transient reaction, as a function of the initial relative concentration of ferricytochrome *c*, $[c^{3+}]_o/[aa_3]$. Various concentrations of oxidase $[aa_3]$ between 2 μM and 4 μM were used. The ferricytochrome *c* concentration was varied between 1 μM and 30 μM. $[c^{2+}]_B$ was obtained as indicated in fig.1. Other conditions were as described in fig.1. The drawn curve is calculated assuming the equilibria described in the eqns. (1) and (2), $K_1 = 3$ and $K_2 = 1$ (see text).

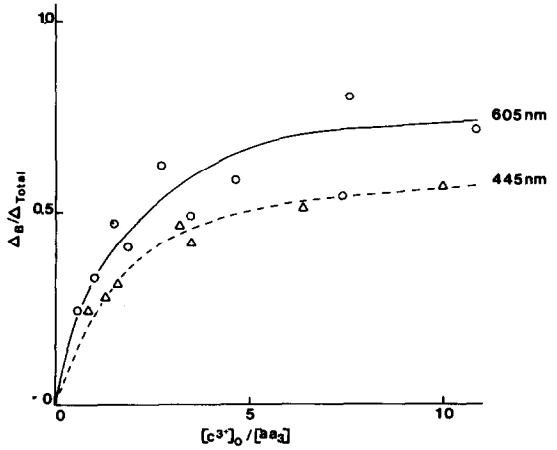


Fig.3. The change in absorption at 605 nm and 445 nm during the rapid reaction compared to the total reduced minus oxidized absorption difference plotted against the initial relative concentration of ferricytochrome *c*, $[c^{3+}]_B/[aa_3]$. The conditions were as described in fig.2. The changes in absorbance at 605 nm and 445 nm are not corrected for changes in cytochrome *c* absorbance.

molecule transferred to cytochrome *c* during the burst phase, $[c^{2+}]_B/[aa_3]$, is plotted against the ratio of initial ferricytochrome *c* to oxidase, $[c^{3+}]_0/[aa_3]$. The number of electrons transferred increases with increasing ferricytochrome *c* concentration but reaches a saturation level of 2 electrons at high relative concentrations of cytochrome *c*. The same maximum of 2 electrons transferred per oxidase molecule was obtained when another enzyme preparation (also of van Buuren type) was used, but the shape of the saturation curve was slightly different.

The amount of heme *a* oxidized in the burst phase monitored by the change in absorbance at 605 nm and at 445 nm is shown in fig.3. A 50% decrease at 445 nm and a 60–70% decrease at 605 nm is observed at high cytochrome *c* concentrations.

The rapid-freeze technique was used to stop the reaction 40 msec after mixing and the e.p.r. spectrum of the intermediate oxidation products obtained by this method was studied. Fig.4 shows the e.p.r. spectrum obtained with various ferricytochrome *c* concentrations and a spectrum of the oxidized enzyme mixed with ferricytochrome *c* under aerobic conditions. The most pronounced effect of the oxidation by cytochrome *c* of the oxidase is the appearance of a high-spin heme signal at g = 6 with both axial and rhombic components of the same appearance as seen in titration experiments [14], and an oxidation of the species, presumably copper, responsible for the signal at g = 2. The signal at g = 3 seems almost exclusively to be due to the low-spin iron of non-reduced cytochrome *c*, since very little, if any, of the low-spin heme signal observed in the fully oxidized enzyme, e.g. the line at g = 1.45 can be detected in this oxidation product of the oxidase (table 1).

The concentrations of the e.p.r.-detectable components oxidized by cytochrome *c* are shown in table 1.

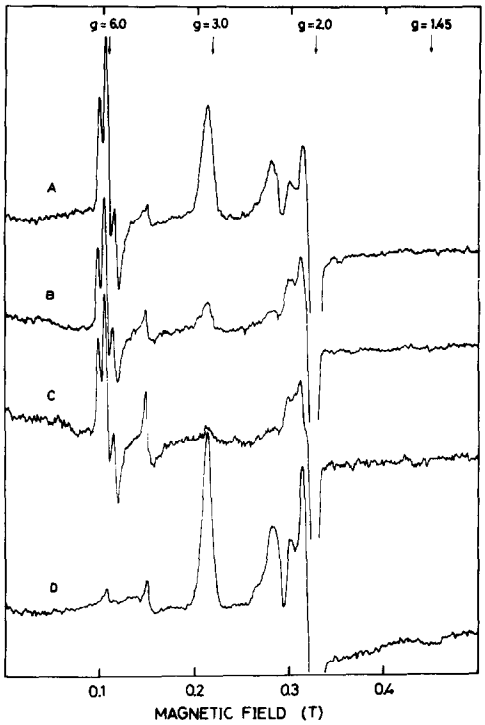


Fig.4. E.p.r. spectra of samples obtained by mixing reduced cytochrome *c* oxidase with ferricytochrome *c*. Rapid-freeze technique was used to quench the reaction after about 40 msec. Reduced cytochrome oxidase, 160 μM in 0.1 M potassium phosphate, pH 7.4, 0.5% Tween-80, was mixed with (A) 5; (B) 1 and (C) 0.5 electron equivalents of ferricytochrome *c*, respectively. Spectrum (D) was obtained after mixing 160 μM of oxidized oxidase with 5 electron equivalents of ferricytochrome *c* in the rapid-freeze apparatus. Microwave frequency, 9.14 GHz; microwave power, 2 mW; modulation amplitude $2 \cdot 10^{-3}$ T; temperature, 18°K. Relative detector gain, 1.0 in (A), (B) and (D); 1.25 in (C).

Table 1
Concentration of e.p.r.-detectable components present 40 msec after mixing of cytochrome *c* oxidase with ferricytochrome *c*

State of aa_3 before mixing	$[c^{3+}]_0/[aa_3]$ ratio	g = 3 (μM)	g = 3 (equivalents per oxidase[a])	g = 1.45 (μM)	g = 6 (μM)	g = 2 (μM)
Oxidized	5	226	5.6	31	–	56
Oxidized	0.5	63	1.5	36	–	58
Reduced	5	138	3.4	<10	10	19
Reduced	1	29	0.7	<10	7	13
Reduced	0.5	11	0.3	0	5.5	9
Reduced	0[b]	–	–	–	–	8

[a]Based on $[aa_3] = 40\ \mu M$.
[b]Mixed with anaerobic buffer.
The conditions are those described in fig.4. The concentration of the g = 3, g = 1.45 and g = 6 components are calculated from the spectra shown in fig.4, while the concentration of the g = 2 component is calculated from e.p.r. spectra of the same samples at 77° K.

The amount of cytochrome *c*, which has been reduced during the rapid reaction, can be estimated from the low-spin signal at g = 3 assuming that the oxidized heme of the oxidase does not appear as detectable low-spin iron (above). After mixing, 3.4 equivalents of cytochrome c^{3+} per oxidase of the 5 initially present are found, indicating that 1.6 equivalents per oxidase are reduced in fair agreement with the value expected from the stopped-flow experiments (see fig.2). The amount of cytochrome *c* reduced at lower $[c^{3+}]_0/[aa_3]$ ratios is however less than expected.

4. Discussion

The steady-state kinetics of the cytochrome *c* oxidase reaction seem to be explained in the most simple way by Minnaerts mechanism IV [15,16]. This mechanism implies the on-constant for the reaction of the enzyme with the substrate, ferrocytochrome *c* to be equal to the on-constant for the reaction with the product, ferricytochrome *c*. The validity of this assumption is supported by the measurements of the transient kinetics of the reaction with ferricytochrome *c* reported in this paper. Evidently, the two reactions are similar with respect to the magnitude of the rate constant. It is also clear that the transient reaction with ferricytochrome *c* is strongly dependent on the ionic strength of the medium in accordance with, what has been found for the reaction with ferrocytochrome *c* [17].

From direct measurements of the reaction between the oxidized enzyme and ferrocytochrome *c* made in this laboratory [5] it was concluded that maximally one electron per oxidase is transferred in the initial fast reaction. A further study of this reaction [7] strongly indicated that a quasi-equilibrium between cytochrome *c* and an electron acceptor of the oxidase is established during the transient phase. The most likely candidate for that role was pointed out to be cytochrome *a*, the potential of which was found to be 285 mV under these circumstances [5,7]. The corresponding measurements of the reaction of the reduced oxidase with ferricytochrome *c* (fig.2) show that the two – and not one electron – are involved. The assumption that the simple equilibrium described by the equations (1) and (2) occur as a result of the transient reaction is sufficient to account for the relation between the burst reduction of cytochrome *c* and the initial cytochrome *c* concentration shown in fig.2,

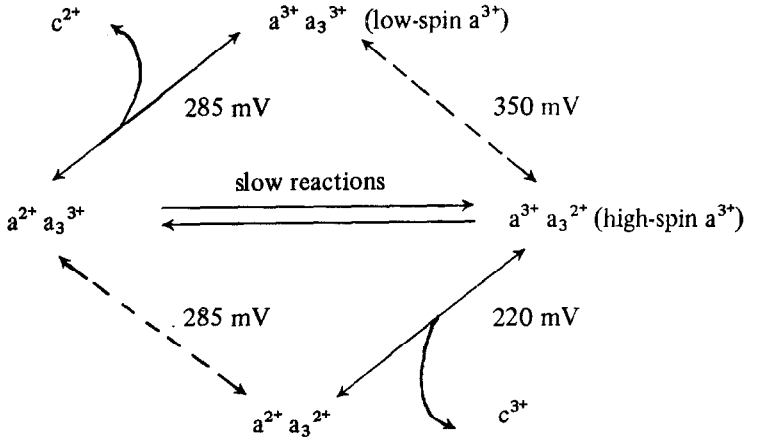
$$c^{3+} + a^{red} \underset{}{\overset{K_1}{\rightleftharpoons}} c^{2+} + a^{ox} \quad (1)$$

$$a^{ox} + b^{red} \underset{}{\overset{K_2}{\rightleftharpoons}} a^{red} + b^{ox} \quad (2)$$

In these equations a and b represent one-electron acceptor groups of the oxidase. A curve calculated for the equilibrium constants $K_1 = 3$ and $K_2 = 1$ is included in fig.2 as the full line. These constants correspond to the existence of two acceptors of equal potential (225 mV, assuming that the potential of cytochrome *c* is 255 mV).

The quasi-equilibrium, determined by the amount of cytochrome *c* reduced in the burst, was observed to be unaffected by the ionic strength of the medium. This observation is most easily explained if it is assumed, in accordance with the mechanism IV of Minnaert [15], that a change in the redox state of cytochrome *c* does not induce any change in the binding constant, K_D, for the complex with the oxidase. A strong dependency on ionic strength of K_D will in that case not be reflected in the overall equilibrium given by equations 1 and 2.

In order to account for the potentials estimated from the stoichiometry of the transient kinetics as well as the potentials obtained from potentiometric titrations [18–20] we propose the following scheme for consideration:



Two aspects of heme–heme interaction, the potentials of cytochrome *a* and a_3 and the spin state of cytochrome *a*, are included in the scheme. The midpoint potential of cytochrome *a* depends on the redox state of cytochrome a_3 being 285 mV when it is oxidized and 220 mV when it is reduced. Such behaviour of cytochrome *a* has previously been proposed by Nicholls [21], see also Nicholls and Petersen [22]. It is in fact an aspect of the dimer model proposed by Tiesjema et al. [20] even though other aspects make this model less attractive, as discussed by Malmström [2].

Both the changes in the optical and the e.p.r. spectrum associated with the reaction are consistent with the notion that heme is being oxidized in the cytochrome oxidase in addition to copper. Low spin cytochrome *a* is rapidly reduced by ferrocytochrome *c* [23,24] in the oxidized enzyme. If cytochrome *a* is assumed to be the site of cytochrome *c* interaction also in the reduced enzyme, it is reasonable to suggest that this site is responsible for the rapidly emerging high-spin signal in the reaction with ferricytochrome *c*. This suggestion is in accordance with the proposal put forward by Hartzell and co-workers [24–26]. They suggested that the bulk of both detectable heme signals ($g = 3$ and $g = 6$) of the oxidase is due to cytochrome *a*, the heme being in a low spin state when cytochrome a_3 is oxidized and in a high-spin state when it is reduced. The interpretation that cytochrome *a*, not cytochrome a_3, is oxidized by cytochrome *c* is supported by our preliminary experiments (not shown) where the CO-complex of the reduced cytochrome *c* oxidase was oxidized by cytochrome *c*. The same stoichiometry and the same rate constant for the reaction with ferricytochrome *c* was found whether the free enzyme or the CO-complex was used.

The role of Cu in the redox reactions of the oxidase is not specified by the above scheme, but the

assumption of a Cu–Cu interaction, or possibly Cu–heme interaction, might be necessary to explain why Cu^{+} is oxidized by ferricytochrome *c* when Cu^{2+} is apparently not [5], or to a smaller degree [24], reduced by ferrocytochrome *c* in the transient reaction.

There is some difficulty in explaining the low recovery of oxidized sites in the e.p.r. experiments in relation to the amount of reacting cytochrome *c*. The difference in the amount of reduced cytochrome *c* found between optical and e.p.r. experiments may however be due to an effect of the temperature on the equilibria in connection with the freezing of the samples. A difference in the reaction might also be considered due to the much higher concentrations used in the e.p.r. experiments.

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