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Effects of cytochrome *c* on the oxidation of reduced cytochrome *c* oxidase by hydrogen peroxide

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Abstract

The oxidation of the redox centres in reduced cytochrome *c* oxidase by hydrogen peroxide was studied by stopped-flow spectrophotometry in the absence and presence of reduced cytochrome *c*. The oxidation rate of cytochrome *a* decreased in the presence of cytochrome *c*. This effect was more pronounced at low than at high ionic strength. Cytochrome *c* did not influence the time-course of the oxidation of Cu_A or cytochrome a_3. The oxidation of cytochrome *c* itself was faster at low ionic strength. The results suggest that the effect of cytochrome *c* is caused by re-reduction of cytochrome *a* by cytochrome *c*, the rate of which is dependent upon the ionic strength. We conclude that cytochrome *a* and cytochrome *c* are in equilibrium and that the equilibrium constant depends on the ionic strength. At low ionic strength as a complex is formed between cytochrome *c* and cytochrome *c* oxidase, cytochrome *a* is more reduced than at high ionic strength conditions, when no such complex exists. Since Cu_A is oxidized at the same rate whether cytochrome *c* is present or not, we conclude that electron transfer from cytochrome *a* or cytochrome *c* to Cu_A is slower than electron transfer from Cu_A to cytochrome *a* or/and to the cytochrome a_3-Cu_B couple.

Key words: Cytochrome *c* oxidase; Hydrogen peroxide; Cytochrome *c*; Ionic strength; Presteady-state kinetics

1. Introduction

Cytochrome *c* oxidase, the last enzyme in the respiratory chain, catalyses the oxidation of cytochrome *c* by oxygen reducing the latter to water. It contains at least four metal centres. Cytochrome *a* and a copper, Cu_A, are in close redox equilibrium and accept electrons from cytochrome *c*. Two electrons can be accepted and these are transferred to the haem a_3-Cu_B couple. This couple binds oxygen and reduces oxygen by twice transferring two electrons. It is still controversial whether cytochrome *a* or Cu_A is the primary acceptor of electrons from cytochrome *c*.

A general model of the electron transfer steps to and in cytochrome *c* oxidase is shown in Fig. 1. The electrons from cytochrome *c* might enter the enzyme via either Cu_A or cytochrome *a* or both. Several electron pathways have been suggested for electron transfer from cytochrome *a* and from Cu_A to the oxygen-binding site. Two electrons, one from Cu_A and one from cytochrome *a*, may go one by one or both at the same time to the a_3-Cu_B couple. In the former case, there are two pathways for internal electron transfer. It is also conceivable that the electron present on cytochrome *a* (as the first electron acceptor) is transferred via Cu_A or alternatively the electron from Cu_A (as the first electron acceptor) is transferred via cytochrome *a* to cytochrome a_3 and Cu_B. Thus, a single pathway for electron flow may also occur.

Since peroxide intermediates exist [1,2] in the oxygen-reduction reaction the study of the oxidation of cytochrome *c* oxidase by hydrogen peroxide is of interest. Gorren et al. [3–5] studied this reaction in detail and the results showed that the internal electron transfer is dependent upon the hydrogen peroxide concentration. At low concentration the rates of oxidation of cytochrome *a* and Cu_A are only 0.5 to 5 s^{-1} [3] increasing with the hydrogen peroxide concentration [4]. At high concentration of hydrogen peroxide (more than 20 mM) these rates increase linearly with the hydrogen peroxide concentration ($k = 700$ $M^{-1}s^{-1}$) [5]. It was

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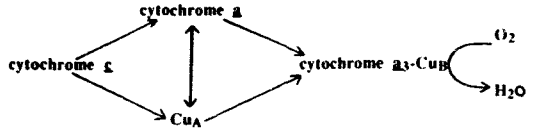

Fig. 1. General scheme of the electron pathways to, in and from cytochrome *c* oxidase. The following steps might occur: electron entry from cytochrome *c* might be at cytochrome *a* or at Cu_A or at both sites; electron transfer from cytochrome *a* and Cu_A to the cytochrome a_3-Cu_B site might occur via two independent or dependent pathways (two electron are transferred simultaneously) or there might also be only one pathway to the cytochrome a_3-Cu_B couple, either one from Cu_A or one from cytochrome *a*.

concluded that binding of hydrogen peroxide to cytochrome a_3 strongly stimulates internal electron transfer. The mechanism of this stimulation is not yet clear. It may be caused either by redox potential changes or conformation changes [5].

Even at these high concentrations of hydrogen peroxide the internal electron transfer was not as fast as in the reaction with oxygen. Since it has been reported that cytochrome *c* oxidase reaches its maximal turnover rate only in the presence of both its substrates [6,7], it was of interest to study the oxidation of cytochrome *c* oxidase by hydrogen peroxide in the presence of cytochrome *c*.

The steady-state reaction as well as the presteady-state reaction of cytochrome *c* oxidase (oxidized) with reduced cytochrome *c* has been studied extensively by several research groups. The second-order rate constant for cytochrome *c* oxidation has been reported to vary from 10^6 $M^{-1}s^{-1}$ at high ionic strength [8–11] to $5 \cdot 10^7$–$2 \cdot 10^8$ $M^{-1}s^{-1}$ at low ionic strength [11,12]. At low ionic strength a stable complex between cytochrome *c* and cytochrome *c* oxidase is formed [13]. It has been reported that the redox potential of cytochrome *c* is decreased by 30 mV as it is bound to cytochrome *c* oxidase [14]. Thus, it was concluded [14] that at low ionic strength electron transfer from cytochrome *c* to cytochrome *c* oxidase is faster than at high ionic strength when cytochrome *c* is not tightly bound. The reaction of reduced cytochrome *c* oxidase with oxidized cytochrome *c* has been studied also under anaerobic conditions. The rate constants were shown to depend on the concentration of reduced cytochrome *a*. A value of $6 \cdot 10^6$ $M^{-1}s^{-1}$ was found at high ionic strength, that increased at lower ionic strength [15].

It is not yet clear whether cytochrome *c* donates its electrons to cytochrome *a* [16,17] or to Cu_A [18,19]. In determinations of the redox-equilibrium constant between cytochrome *c* and cytochrome *c* oxidase it varies from 0.5 or 1.0 [20] to 3 [7,15]. The redox potential of cytochrome *a* and Cu_A are about the same corresponding to an equilibrium constant of 1 [7] or 0.7 [21]. The redox potential difference between cytochrome *c* and cytochrome *a* of about 30 mV [14] would correspond to an equilibrium constant of 3. Recently the oxidation of reduced cytochrome *c* oxidase by oxygen in the presence of cytochrome *c* at low ionic strength has been described by Hill [22]. It was concluded that Cu_A is the primary electron acceptor and that cytochrome *a* mediates electron transfer from Cu_A to the cytochrome a_3-Cu_3 couple.

The equilibration between cytochrome *a* and Cu_A has been suggested to be very fast. Rates that have been reported for the equilibration vary from 18 s^{-1} [16], 50 s^{-1} [17,23], 400 s^{-1} [24] to 2500 s^{-1} [25]. Morgan et al. [21] observed a very rapid re-equilibration rate (17000 s^{-1}) between cytochrome *a* and Cu_A in studies of three-electron-reduced carbon-monoxide-bound cytochrome *c* oxidase. It may be that, as suggested by Fabian et al. [9], the state of the enzyme affects the rates of electron transfer between Cu_A and cytochrome *a*.

The reaction of oxygen with cytochrome *c* oxidase, which is extremely fast, has been studied extensively. Oxygen binds to the cytochrome a_3-Cu_B couple with a rate constant of 10^8 $M^{-1}s^{-1}$ [17,26,27]. Several intermediates of oxygen reduction are suggested to exist [1,26,28–33]. The rates found for the formation of these intermediates vary from 300 s^{-1} [26] to 10^5 s^{-1} [33]. The rates found for internal electron transfer from cytochrome *a* or Cu_A to cytochrome a_3 vary from 5 s^{-1} to 10^4 s^{-1} and are explained with many different models [8,9,17,24,27,33,34]. Some models incorporate conformation changes of cytochrome *c* oxidase during redox cycling [9]. Some of the measured rates differ just because the experiments were carried out with 'slow' or 'fast' forms of cytochrome *c* oxidase [17,35].

In addition to several studies where hydrogen peroxide was used to study the formation of intermediates [28,36,37], the peroxidase-activity of cytochrome *c* oxidase (the oxidation of reduced cytochrome *c* by hydrogen peroxide) has been studied [10,32,38]. Reduction of hydrogen peroxide to water is about ten times slower than oxygen reduction [38]. It has been described that the addition of hydrogen peroxide inhibits oxygen consumption in a polarographic measurement [38]. Orii [10,32] describes that, due to peroxidase activity at the same time as oxidase activity, cytochrome *c* oxidation at low oxygen concentrations was accelerated in the presence of hydrogen peroxide.

Hydrogen peroxide is a two-electron acceptor. Reduced cytochrome a_3 and Cu_B are oxidized rapidly ($2 \cdot 10^4$–$3 \cdot 10^4$ $M^{-1}s^{-1}$ [3,4,5]) by hydrogen peroxide and after the initial oxidation of the a_3-Cu_B site a second hydrogen peroxide has to bind to this site resulting in oxidation of cytochrome *a* and Cu_A. Eventually, a third hydrogen peroxide will bind to the oxidized cytochrome a_3-Cu_B site. In the presence of re-

duced cytochrome *c* in a ratio of one molecule of cytochrome *c* to one molecule of cytochrome *c* oxidase, the enzyme system contains five electrons at the start of the reaction. Thus, when reoxidation by two hydrogen peroxide molecules is completed one electron remains and will be present on cytochrome *c*, cytochrome *a* or Cu_A. In principle this approach opens the possibility to study the effects of cytochrome *c* on the oxidation rates of cytochrome *a* and Cu_A.

2. Materials and methods

Cytochrome *c* oxidase was isolated from bovine heart according to [39]. The catalase activity of the cytochrome *c* oxidase preparation used in this study was negligible [5]. Cytochrome *c* was isolated from horse heart according to [40] or was purchased from Sigma, USA (horse heart type VI). The concentrations were determined spectrophotometrically using an ϵ_{550nm}(red-ox) of 21 $mM^{-1}cm^{-1}$ for cytochrome *c* and an ϵ_{605nm}(red-ox) of 24 $mM^{-1}cm^{-1}$ for cytochrome *c* oxidase. Glucose oxidase (*Aspergillus niger*, grad. II) was from Boehringer Mannheim GmbH, F.R.G. All other chemicals were of analytical grade and purchased from Merck (Darmstadt, F.R.G.) or BDH (UK).

The measurements were carried out using a Union Giken stopped-flow spectrophotometer RA401. The experiments were carried out at 20°C in buffers of pH 7.4 with 0.5 or 1% Tween-80 and 50 mM glucose at high ionic strength (100 mM potassium phosphate) or at low ionic strength (5 mM potassium phosphate). Before mixing, the reactants were incubated in the stopped-flow vessels under a 5 atm N_2 pressure for about 10 min in order to be sure that cytochrome *c* oxidase and cytochrome *c* were fully reduced and anaerobiosis was reached. One vessel was made anaerobic by adding 500 μM sodium dithionite and cytochrome *c* oxidase and cytochrome *c*, when present, were added to this vessel and thus were reduced. The other vessel was made anaerobic by addition of 250 units of glucose oxidase and hydrogen peroxide was added to this vessel. After mixing both vessels, the concentration of cytochrome *c* oxidase and of cytochrome *c*, when present, was 5 μM each and the concentration of hydrogen peroxide ranged from 125 μM to 20 mM. This method has been described previously [4,5,38].

The oxidation of the prosthetic groups was measured optically; cytochrome *c* at 550 nm, cytochrome *a* at 605 nm, Cu_A at 830 nm and cytochrome a_3 at 436 nm. At 550 nm, the wavelength used to measure oxidation of cytochrome *c*, negligible interference with absorbance changes of cytochrome *c* oxidase takes place. At 605 nm cytochrome a_3 has also some absorbance, but the extinction coefficient is much lower than that for cytochrome *a*. Moreover, the rate of oxidation of cytochrome a_3 is much higher than that of cytochrome *a* at the high hydrogen peroxide concentrations used in our experiments and thus, absorbance changes of both cytochromes could be clearly distinguished at 605 nm. Finally, a complex of oxidized cytochrome a_3 and hydrogen peroxide is formed which results in an absorbance increase at this wavelength [41]. The formation of the hydrogen peroxide complex also causes an absorbance increase at 436 nm. The rate of complex formation, however, is much lower than the oxidation rate of cytochrome a_3 and it is formed after cytochrome a_3 is fully oxidized [5]. The absorbance changes at 830 nm are all due to Cu_A under the experimental conditions of the present study. Since the extinction coefficient of Cu_A is low compared to the extinction coefficients of the other species measured, the results for Cu_A are less accurate with a variance of about 5%, whereas the errors in the results of the other species are smaller than 1%.

The rate constants were calculated by using the first-order fitting programme with which the apparatus is equipped. The Union Giken RA415 computer and the programme called SF.SAV were used. The rates vary during the reaction, since after cytochrome *c* oxidase is partly oxidized re-reduction by cytochrome *c* will occur. Since dithionite reacts very rapidly with oxidized cytochrome *c* and the re-reduced cytochrome *c* can reduce cytochrome *c* oxidase again, interference from dithionite was found in the later stages of the reaction. In the initial part of the reaction, when most of the cytochrome *c* present is still reduced, the interference is negligible.The plot of the logarithm of absorbance versus time is linear in that phase. Therefore, the rate constants of the initial phase of the reaction were determined.

3. Results

Fig. 2 shows that the observed rate of oxidation of cytochrome a_3 depends linearly on the hydrogen peroxide concentration. A second-order rate constant of $1.5 \cdot 10^4$ $M^{-1}s^{-1}$ was calculated from the slope of the line. This line fits the data obtained at all measured conditions, i.e., in the absence of cytochrome *c* both at high and at low ionic strength and in the presence of cytochrome *c* at high ionic strength as well as at low ionic strength when cytochrome *c* and cytochrome *c* oxidase are present as a 1 to 1 complex. The value for the rate constant is in agreement with that found previously in the absence of cytochrome *c* [5]. Thus, cytochrome *c* has no effect on the oxidation rate of cytochrome a_3 by hydrogen peroxide.

Before the observed oxidation rates of Cu_A, cytochrome *a*, and cytochrome *c* by hydrogen peroxide

are presented, the results of some control experiments are reported. A side reaction of dithionite consuming the substrate hydrogen peroxide can be neglected, since hydrogen peroxide reacts only very slowly with dithionite.

Cytochrome *c* oxidase and the dissolved oxygen present in the solution of reactants that was put in one vessel were reduced by a small excess of dithionite (cf. Materials and Methods). Since the reduction rate of cytochrome *c* oxidase by dithionite is only $8 \cdot 10^4$–$16 \cdot 10^4$ $M^{-1}s^{-1}$ [42,43], hardly any re-reduction of cytochrome *c* oxidase by dithionite occurs in the measuring time of our experiments [4].

In the presence of cytochrome *c*, however, the slight excess of dithionite will re-reduce this component rapidly and electron transfer may occur between re-reduced cytochrome *c* and cytochrome *c* oxidase. In control experiments we measured the reduction rate of oxidized cytochrome *c* by dithionite. Cytochrome *c* was present in the vessel that was made anaerobic by glucose oxidase and thus remained fully oxidized. The other vessel, made anaerobic by dithionite (500 μM), contained no cytochrome *c* or cytochrome *c* oxidase. After mixing both vessels the concentration of cytochrome *c* was either 2.5 or 5.0 μM. A rate of 17 s^{-1} was found for the reduction of 5.0 μM cytochrome *c* and 2.5 μM cytochrome *c* was reduced at a rate of 14 s^{-1}. In this reaction cytochrome *c* is reduced by the small excess of dithionite that remains after anaerobiosis was reached. The rate depends on the dithionite concentration as well as on the concentration of cytochrome *c*. It should be noted that, under the conditions of the experiments proper, much lower concentrations of oxidized cytochrome *c* are present, since the reaction starts with fully reduced cytochrome *c* present in the other vessel.

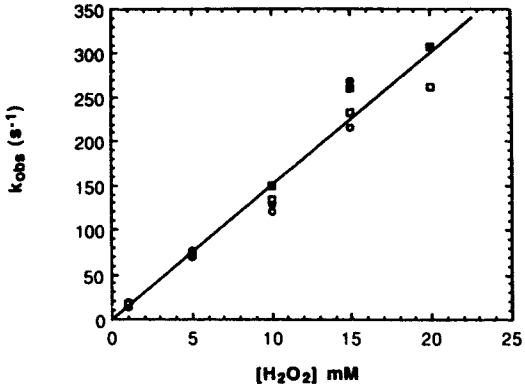

Fig. 2. Dependence of the observed rate constant for the oxidation of cytochrome a_3 on the hydrogen peroxide concentration. The reaction was measured at 436 nm under different conditions: without cytochrome *c* present at high ionic strength (closed circles) and at low ionic strength (open circles) or in the presence of cytochrome *c* at high ionic strength (closed squares) and at low ionic strength (open squares). The concentration of cytochrome *c* oxidase and of cytochrome *c* was 5 μM. The experimental conditions are described in detail in the Materials and Methods section.

Table 1
The percentage of Cu_A, cytochrome *a* and cytochrome *c* that is oxidized in 500 ms after mixing reduced cytochrome *c* oxidase (5 μM) with 5 mM H_2O_2

Conditions			% oxidized		
[Cyt. *c*] (μM)	ionic strength	[dithionite] (μM)	Cu_A	cytochrome *a*	cytochrome *c*
[5]	high	350	50	42	40
		700	52	35	8
	low	400	85	89	55
		600	78	46	38
[0]	high	500	75	68	
	low	500	66	73	

The percentage oxidation was measured under several conditions; in the presence of cytochrome *c* (5 μM) at high ionic strength with a final added concentration of 350 μM or 700 μM dithionite; at low ionic strength with 400 μM or 600 μM dithionite; in the absence of cytochrome *c* at a dithionite concentration of 500 μM, at both high and low ionic strength. The amount of each species was calculated from the absorbance at 500 ms minus that at 0 ms divided by the extinction coefficient. The total concentration present during the reaction was taken as 100%. The variance on the data of Cu_A is about 5%, whereas the error on the other data is smaller than 1%.

The non-catalytic oxidation of dithionite-reduced cytochrome *c* with different concentrations of hydrogen peroxide was studied as a control (results not shown). Even at the highest concentration of hydrogen peroxide used (20 mM), cytochrome *c* was oxidized very slowly (in the order of minutes). Thus, the time scale of this reaction is too long to interfere with our measurements.

In order to check the influence of dithionite on the oxidation of cytochrome *c* oxidase and cytochrome *c* by hydrogen peroxide, experiments were carried out at several concentrations of dithionite which were both higher and lower than 500 μM. It was observed that at least a concentration of 350 μM (at high ionic strength) or 400 μM (at low ionic strength) was required for full reduction of the reaction components.

Table 1 shows the effect of ionic strength and dithionite on the oxidation level of Cu_A, cytochrome *a*, and cytochrome *c* upon reoxidation by 5 mM hydrogen peroxide after 500 ms. As a comparison also the percentages of oxidation of cytochrome *a* and Cu_A in the absence of cytochrome *c* at a dithionite concentration of 500 μM are presented. Since there was no effect of dithionite on the oxidation rate of cytochrome a_3 the results for this component are not shown in Table 1.

At a dithionite concentration of 350 μM at high ionic strength 40% of the cytochrome *c* is oxidized in a fast phase, whereas at higher dithionite concentration

cytochrome *c* remains almost completely reduced. Neither the level of oxidation of cytochrome *a* nor that of Cu_A were significantly affected by a higher concentration of dithionite at high ionic strength.

At low ionic strength, when cytochrome *c* and cytochrome *c* oxidase form a stable 1:1 complex, the amount of cytochrome *a* oxidized is markedly affected by the higher concentration of dithionite. In contrast, the redox levels of cytochrome *c* and of Cu_A are not significantly altered in the presence of a higher concentration of dithionite at low ionic strength. Apparently, under these conditions re-reduction of cytochrome *a*, but not of Cu_A, occurs via cytochrome *c*.

The level of oxidation of cytochrome *a* and of Cu_A at a dithionite concentration of 350–400 μM in the presence of cytochrome *c* is about twice as large at low ionic strength than at high ionic strength (Table 1). At high ionic strength some kind of steady-state level is already reached after about 300 ms whereas at low ionic strength the steady-state level is reached later and consequently, cytochrome *a* and Cu_A are more oxidized after 500 ms (results not shown). This pseudo-steady state is reached when the rates of oxidation and of re-reduction of cytochrome *a* and of Cu_A are the same. There are two possibilities to explain the difference between the results at high ionic strength and at low ionic strength. First, internal electron transfer could be faster when cytochrome *c* is bound to cytochrome *c* oxidase (at low ionic strength) than when it is not bound (at high ionic strength). Secondly, re-reduction of cytochrome *c* could be faster at high ionic strength than at low ionic strength. It seems that, although the observed rate of oxidation of Cu_A is not affected by cytochrome *c* (Fig. 3), the oxidation of Cu_A is faster at low ionic strength (when cytochrome *c* is bound to cytochrome *c* oxidase) and that at high ionic strength Cu_A is re-reduced by cytochrome *c* probably via cytochrome *a*. However, after the pseudo-steady state has been passed further oxidation of cytochrome *a*, but not of Cu_A, occurs.

Gorren et al. [5] showed that the observed rate of oxidation of cytochrome *a* and of Cu_A depends on the hydrogen peroxide concentration. At high concentrations of hydrogen peroxide (20–45 mM) they found a linear increase of the rates with the concentration of H_2O_2. Fig. 3 shows the rate of oxidation of Cu_A as a function of the hydrogen peroxide concentration. It is obvious that the oxidation rate of Cu_A is not affected by the presence of cytochrome *c* or by the ionic strength.

In contrast to the result found for Cu_A as shown in Fig. 3, the observed oxidation rate of cytochrome *a* is decreased in the presence of cytochrome *c* (Fig. 4). This effect is larger at low ionic strength than at high ionic strength. In the absence of cytochrome *c* the same dependence on the concentration of H_2O_2 is

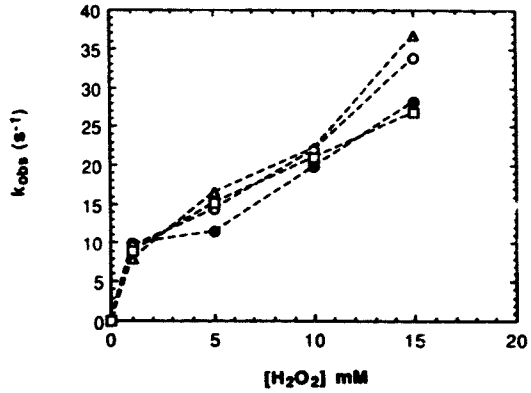

Fig. 3. Dependence of the observed rate constant for the oxidation of Cu_A on the hydrogen peroxide concentration. The reaction was measured at 830 nm at different conditions: without cytochrome *c* present at high ionic strength (closed circles) and at low ionic strength (open circles) or in the presence of cytochrome *c* at high ionic strength (triangles) and at low ionic strength (squares). The concentration of cytochrome *c* oxidase and of cytochrome *c* was 5 μM. The experimental conditions are described in detail in the Materials and Methods section.

found for cytochrome *a* as for Cu_A, both at high and at low ionic strength [6]. It is obvious from a comparison of the data in Fig. 3 and Fig. 4 that cytochrome *c* has a significant effect on the observed rate constants of oxidation of cytochrome *a*, but not on that of Cu_A.

Some measurements of cytochrome *a* oxidation were carried out at 428 nm (results not shown), since neither cytochrome a_3 nor the cytochrome a_3-H_2O_2 complex

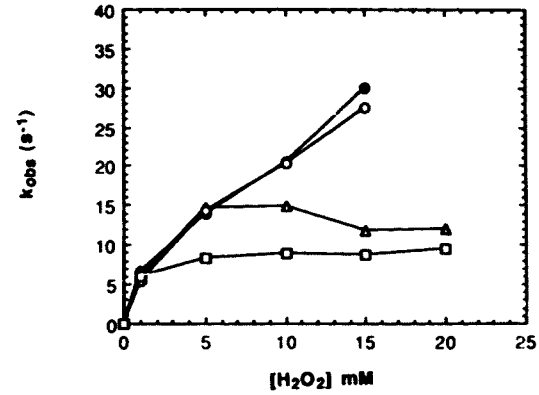

Fig. 4. Dependence of the observed rate constant for the oxidation of cytochrome *a* on the hydrogen peroxide concentration. The reaction was measured at 605 nm at different conditions: without cytochrome *c* present at high ionic strength (closed circles) and at low ionic strength (open circles) or in the presence of cytochrome *c* at high ionic strength (triangles) and at low ionic strength (squares). The concentration of cytochrome *c* oxidase and of cytochrome *c* was 5 μM. The experimental conditions are described in detail in the Materials and Methods section.

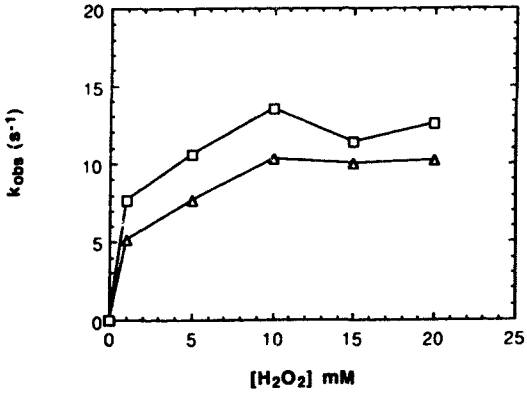

Fig. 5. Dependence of the observed rate constant for the oxidation of cytochrome *c* on the hydrogen peroxide concentration. The reaction was measured at 550 nm at high ionic strength (triangles) or at low ionic strength (squares). The concentration of cytochrome *c* oxidase and of cytochrome *c* was 5 μM. The experimental conditions are described in detail in the Materials and Methods section.

interferes at this wavelength (results not shown). Although the rates were a little lower than those obtained from the measurements at 605 nm, the effect of cytochrome *c* on the oxidation rate of cytochrome *a* was the same as shown in Fig. 4.

In the experiments of oxidation of reduced cytochrome *c* oxidase in the presence of reduced cytochrome *c* by hydrogen peroxide at high and at low ionic strength, the rates of oxidation of cytochrome *c* were also measured at 550 nm. The observed rate constants are plotted against the hydrogen peroxide concentration and presented in Fig. 5. At low ionic strength the observed rates are higher than at high ionic strength. A comparison of this result with that in Fig. 4 shows that the effect of ionic strength on the oxidation rate of cytochrome *a* is opposite to that of cytochrome *c*. These results suggest that the decrease of the oxidation rate of cytochrome *a* in the presence of cytochrome *c* is caused by the re-reduction of cytochrome *a* by cytochrome *c*, the rate of which is dependent on ionic strength [12].

4. Discussion

A number of conformations of oxidized cytochrome *c* oxidase have been described in literature. These are: resting (as isolated), pulsed and oxygenated [2,35,38, 44–46]. Since dithionite was added and oxygen is present in our buffers, the enzyme has been in turnover and this has been shown to activate cytochrome *c* oxidase [2]. Furthermore, by reduction of cytochrome *c* oxidase the bond between cytochrome a_3 and Cu_B, that determines whether the enzyme is in an active state, is broken [47]. Thus, in our experiments we most probably deal with an activated form of cytochrome *c* oxidase.

The presence of a slight excess of dithionite may affect our kinetic data, since the rate of reduction of cytochrome *c* by dithionite was found to be $4.6 \cdot 10^7$ $M^{-1}s^{-1}$ [43]. Initially in our experiments only reduced cytochrome *c* was present, suggesting that the effect of re-reduction of cytochrome *c* by dithionite is small under pre-steady-state conditions. On a longer timescale, however, a steady-state redox level of cytochrome *a* and cytochrome *c* was reached. Only after all dithionite was consumed the oxidation was completed. These results are comparable to the results of [41] where a temporary steady state in reduction of cytochrome *a* was reached when cytochrome *c* oxidase was reduced by dithionite in the presence of hydrogen peroxide.

The oxidation levels of cytochrome *a* and Cu_A, as determined at a fixed period after initiation of the reaction and with a very small excess of dithionite in the presence or in the absence of cytochrome *c*, differ slightly. At high ionic strength Cu_A is more oxidized than cytochrome *a* which is in agreement with the finding of Morgan et al. [21] that the redox potential of Cu_A is slightly lower than that of cytochrome *a*. At low ionic strength cytochrome *a* is slightly more oxidized than Cu_A. This suggests that the redox potential of cytochrome *a* or of Cu_A is affected by the salt concentration. With a larger excess of dithionite in the presence of cytochrome *c*, we find a lower oxidation level of cytochrome *a* than of Cu_A. This effect is due to re-reduction of cytochrome *a* and has a kinetic nature, for at high ionic strength the oxidation level of cytochrome *c* was decreased.

The oxidation level of cytochrome *a* and of Cu_A, measured at a fixed period after initiation of the reaction, is much higher when cytochrome *c* forms a complex with cytochrome *c* oxidase (at low ionic strength) than at high ionic strength when cytochrome *c* is not bound. This was found at low dithionite concentration when no significant re-reduction occurs other than re-reduction by reduced cytochrome *c* itself. Two explanations for this difference may be offered: faster internal electron transfer at low ionic strength or faster re-reduction (by a small excess of dithionite) at high ionic strength. It might be that with a low dithionite concentration of 350–400 μM still a slight excess of dithionite is present that re-reduces cytochrome *c* and subsequently cytochrome *a* and Cu_A. The differences in oxidation level can then be explained by the fact that cytochrome *c* is reduced more easily by dithionite when it is not bound to cytochrome *c* oxidase. We suggest that not only re-reduction by cytochrome *c* but also oxidation of Cu_A and cytochrome *a* (internal electron transfer) is faster when cytochrome *c* is bound.

This does not, however, result in higher observed rates of oxidation of cytochrome *a* or Cu_A.

The rate of cytochrome a_3 oxidation by hydrogen peroxide is neither affected by the presence of cytochrome *c* nor by the ionic strength. This indicates that the oxidation of cytochrome a_3 is always faster than the internal electron transfer from cytochrome *a* or Cu_A to the cytochrome a_3-Cu_B couple.

The rate of oxidation of cytochrome *a* is decreased in the presence of cytochrome *c*. This seems to be caused by re-reduction of cytochrome *c* oxidase by cytochrome *c*, since the oxidation rate of cytochrome *c* is faster at low ionic strength than at high ionic strength. This effect is more pronounced when cytochrome *c* is tightly bound (at low ionic strength) than at high ionic strength.

However, the rate of oxidation of Cu_A was affected neither by dithionite nor by the ionic strength of the medium. The rate of oxidation of Cu_A showed the same relation to the concentration of hydrogen peroxide as has been described [6] for the internal electron transfer from cytochrome *a* and Cu_A to the cytochrome a_3-Cu_B couple in the absence of cytochrome *c*. This was not found by Hill [22]. He observed that oxidation of cytochrome *a* as well as oxidation of Cu_A is affected in the presence of bound cytochrome *c* (at low ionic strength). However, in his study oxygen was the (four-)electron acceptor, whereas in our studies hydrogen peroxide, a two-electron acceptor, was used.

For the oxidation of cytochrome *c* by cytochrome *c* oxidase rates of $5 \cdot 10^7$–$2 \cdot 10^8$ $M^{-1}s^{-1}$ at low ionic strength (0.01 M) and rates of around 10^6 $M^{-1}s^{-1}$ at high ionic strength (about 0.3 M) were reported [8–12]. Under the experimental conditions used in our study, rates of 250–1000 s^{-1} at low ionic strength and of around 5 s^{-1} at high ionic strength can be calculated. However, the oxidation rate of cytochrome *c* is also dependent on the concentration of oxidized cytochrome *a* [15]. Since under our experimental conditions the reaction starts with cytochrome *a* completely reduced, the actual rate of oxidation of cytochrome *c* might be lower. The rate of oxidation of cytochrome *a* is decreased in the presence of cytochrome *c*. Since oxidation occurs in the initial phase, the oxidation of cytochrome *a* is faster than the re-reduction by cytochrome *c* both at high and at low ionic strength. Otherwise one would expect cytochrome *a* to remain completely reduced.

The results for the oxidation of Cu_A clearly show that the electron transfer from this component to the cytochrome a_3-Cu_B site (or to cytochrome *a*) is always faster than electron transfer from cytochrome *a* (or cytochrome *c*) to Cu_A under the conditions of the experiments presented here. This indicates that there is no fast electron transfer from cytochrome *a* to Cu_A. Considering this slow equilibrium rate between cytochrome *a* and Cu_A and the effect of cytochrome *c* that is found on the oxidation rate of cytochrome *a*, the primary electron acceptor of cytochrome *c* oxidase seems to be cytochrome *a*.

It is also conceivable that Cu_A first accepts the electrons [48], but than electron transfer from Cu_A to cytochrome *a* must be faster than electron transfer from cytochrome *a* to Cu_A. In that case the equilibrium constant between cytochrome *a* and Cu_A should be unequal to 1 and depend on binding of cytochrome *c*. Such an effect is proposed by Brzezinski et al. [8], who concluded that the redox equilibrium between cytochrome *a* and Cu_A changes in the presence of cytochrome *c* at low ionic strength compared to high ionic strength. Our results suggest that the electron transfer from cytochrome *a* to Cu_A is slower than the rate of Cu_A oxidation or at least slower than the electron transfer from cytochrome *c* to cytochrome *a*.

Slow electron transfer from cytochrome *a* to Cu_A, as we conclude, would be in agreement with [11] and [16] and with the model described in [8]. However, our studies show slower electron transfer between cytochrome *a* and Cu_A than from cytochrome *a* and from Cu_A to the cytochrome a_3-Cu_B site, even at lower hydrogen peroxide concentrations. The rates we measure are lower than the rates described in [8], probably because hydrogen peroxide instead of oxygen was used as oxidant. In this respect it should be noted that the turnover of cytochrome *c* oxidase with hydrogen peroxide is ten times slower than the oxidation of cytochrome *c* in the presence of oxygen [38].

Hill et al. [17] presented some results from which they concluded that there is a pathway from cytochrome *c* directly to the a_3-Cu_B couple and oxygen while cytochrome *a* is by-passed. This has been confirmed by Wrigglesworth et al. [42], who propose a conformation change due to binding of cytochrome *c* in such a way that Cu_B can be reduced directly by cytochrome *c*. Our experiments do not show the presence of any such pathway.

The discrepancy in the rates of internal electron transfer between studies in which the carboxy-cytochrome *c* oxidase is used [21,27,33] compared to the results of the experiments reported here may be explained as follows. The high rates found for the reverse electron flows (from a_3-Cu_B to *a* and/or Cu_A) in photodissociated (partly) reduced carboxy-cytochrome *c* oxidase as well as those found for the oxidation of this enzyme form by oxygen may be caused by the massive and very fast drop of the potential of cytochrome a_3 after photolysis of CO. Furthermore, after dissociation CO is rebound to the reduced Cu_B [49] and cytochrome a_3 might be oxidized, which could also affect electron transfer. The couple of cytochrome a_3 and Cu_B plays an important role in oxidation of the reduced enzyme. This role and in particular that of

Cu_B is not well understood. Many mechanisms for the binding of oxygen and its subsequent reduction at this site have been proposed, but these models cannot explain the discrepancy mentioned above. Such an effect of fast potential drop might also occur in the experiments of Morgan et al. [21], who found a very high rate constant for the equilibrium between cytochrome *a* and Cu_A. It is interesting that such high rates can be reached but it is not sure whether this plays a role in the kinetics of cytochrome *c* oxidase with substrates. According to Wikström et al. [50] Cu_A might play a role as an electron buffer under physiological conditions. This could explain the divergence in rates under various experimental conditions. Cu_A then plays various roles in electron transfer and reacts with different rates, depending on the conditions.

In conclusion it can be noted that cytochrome *a* probably is the primary electron-acceptor. Electrons at cytochrome *a* can be transferred either to Cu_A at a relatively low rate or to the cytochrome a_3-Cu_B couple at a rate dependent upon the concentration of hydrogen peroxide. Electron transfer from Cu_A to cytochrome *a* and to the cytochrome a_3-Cu_B couple is faster than re-reduction by either cytochrome *c* or cytochrome *a*.

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