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Rate enhancement of the internal electron transfer in cytochrome *c* oxidase by the formation of a peroxide complex; its implication on the reaction mechanism of cytochrome *c* oxidase

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The oxidation of reduced cytochrome *c* oxidase by hydrogen peroxide was investigated with stopped-flow methods. It was reported by us previously (A.C.F. Gorren, H. Dekker and R. Wever (1986) Biochim. Biophys. Acta 852, 81–92) that at low H_2O_2 concentrations cytochrome *a* is oxidised simultaneously with cytochrome a_3, but that at higher H_2O_2 concentrations the oxidation of cytochrome *a* is slower than that of cytochrome a_3. We now report that for high peroxide concentrations (10–45 mM) the oxidation rate of cytochrome *a* increased linearly with the concentration of H_2O_2 ($k = 700\ M^{-1} \cdot s^{-1}$). Upon extrapolation to zero H_2O_2 concentration an intercept with a value of $16\ s^{-1}$ (at 20 °C and pH 7.4) was found. A reaction sequence is described to explain these results; according to this model the rate constant ($16\ s^{-1}$) at zero H_2O_2 concentration represents the true value of the rate of electron transfer from cytochrome *a* to cytochrome a_3 when the a_3-Cu_B site is oxidised and unligated. However, when a complex of hydrogen peroxide with oxidised cytochrome a_3 is formed, this rate is strongly enhanced. The slope ($700\ M^{-1} \cdot s^{-1}$) would then represent the rate of cytochrome $a_3{}^{3+}$-H_2O_2 complex formation. From experiments in which the pH was varied, we conclude that the reaction of H_2O_2 with cytochrome $a_3{}^{2+}$ is independent of pH, whereas the electron-transfer rate from cytochrome *a* to cytochrome a_3 gradually decreases with increasing pH. From the temperature dependence we could calculate values of $23\ kJ \cdot mol^{-1}$ and $45\ kJ \cdot mol^{-1}$ for the activation energies of the oxidations by H_2O_2 of cytochrome $a_3{}^{2+}$ and cytochrome a^{2+}, respectively. The similarity of the values that were obtained for cytochrome *a* oxidation both with H_2O_2 and with O_2 as the electron acceptor suggests that the reactions share the same mechanism. In ^2H_2O the reactions studied decreased in rate. For the reaction of $^2H_2O_2$ with reduced cytochrome a_3 in ^2H_2O, a small effect was found (15% decrease in rate constant). However, the internal electron-transfer rate from cytochrome *a* to cytochrome a_3 decreased by 50%. Our results suggest that the internal electron transfer is associated with proton translocation.

Introduction

Cytochrome *c* oxidase (ferrocytochrome *c*: oxygen oxidoreductase EC 1.9.3.1) is the terminal enzyme of the mitochondrial respiratory chain. The enzyme contains four prosthetic groups: two haem *a* groups (of cytochrome *a* and of cytochrome a_3) and two copper atoms (Cu_A and Cu_B) [1]. Two of these centres (the haem *a* group of cytochrome *a* and Cu_A) are involved in accepting electrons from cytochrome *c*, whereas the other two (the haem *a* group of cytochrome a_3 and

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Cu_B) form a binuclear reaction site where oxygen binding and reduction takes place [2,3].

Oxidised cytochrome *c* oxidase is known to exist in various forms [4–11]. In this paper we will only discriminate between two of these forms. The 'resting' oxidase, i.e., the enzyme as isolated, is inhomogeneous. It consists of a mixture of different conformations that react at different rates with many ligands and redox reagents [4,9]. The composition of this mixture differs depending upon the isolation method [4,9,10] and is reflected in the variable position of the Soret absorbance maximum. In the preparation we used, which is of the modified Fowler type, the maximum of this peak is at 424 nm [5]. After the oxidase has undergone a catalytic cycle a conformation is formed which is called 'pulsed'. The term was introduced by Antonini et al. [12], who found that shortly after reoxidation the enzyme has a higher catalytic activity than the 'resting' enzyme. Originally, this enzyme form was supposed to be identical to that obtained when cytochrome *c* oxidase is reduced with excess sodium dithionite and subsequently reoxidised by O_2. This enzyme form has a Soret absorbance maximum at 426–428 nm and was called the 'oxygenated' form [5,7,13–15]. However, Kumar et al. [16,17] showed that an enzyme form with the enhanced activity of the 'pulsed' enzyme but with the Soret peak at 420 nm was observed if sufficient precautions were taken to prevent cytochrome *c* oxidase from reacting with hydrogen peroxide. Our studies concerning the complex formation of oxidised cytochrome a_3 with H_2O_2 confirm these results [18]. When in the present paper the term 'pulsed' is used this is done in the sense of Kumar et al. [16,17]. It should be noted, however, that the difference in Soret peak position that exists between the 'resting' cytochrome *c* oxidase forms of their and of our preparations (418 nm and 424 nm, respectively) also exists between the 'pulsed' forms: contrary to the "420 nm 'pulsed' enzyme" of Kumar et al. our pulsed enzyme had an absorbance peak at 424 nm. When in the present paper the term 'pulsed' is used, it refers to the enzyme form that is obtained in stopped-flow experiments by the method that was applied by us previously [18], and that is explained in the Materials and Methods section.

H_2O_2 reacts with both reduced and oxidised cytochrome *c* oxidase. With oxidised cytochrome a_3 it forms a stable complex, a reaction which has been thoroughly invertigated [16,19–21], since a peroxide complex is assumed to be an intermediate in the reaction of reduced cytochrome *c* oxidase with oxygen [22–26]. The complex formation is not monophasic. With the 'pulsed' enzyme, which is probably homogeneous, two phases with different spectral contributions can be observed in the visible region [18,20], whereas in the Soret region the reaction is monophasic [18]. The rate of the first phase is dependent on the peroxide concentration ($k = 700\ M^{-1} \cdot s^{-1}$). With the resting enzyme, which consists of a mixture of different enzyme forms, the reaction with hydrogen peroxide is more complex [18,20]; in the 'resting' enzyme 45% of the oxidase reacts with H_2O_2 at the same rate as the pulsed enzyme ($700\ M^{-1} \cdot s^{-1}$) [18].

With reduced cytochrome *c* oxidase H_2O_2 acts as an electron acceptor [13,19,27,28]. As previously shown by us [18,29], the reaction takes place at the oxygen-binding site. The rate constant, which is independent of the redox state of Cu_A and cytochrome *a*, is $2 \cdot 10^4\ M^{-1} \cdot s^{-1}$. The most striking observation was that the internal electron transfer from cytochrome *a* to cytochrome a_3 was much slower with H_2O_2 as the ultimate electron acceptor (approx. $20\ s^{-1}$) than with O_2 (at least $700\ s^{-1}$) [30,31]. One explanation for the difference in internal electron-transfer rates is that the formation of a peroxide complex at the a_3-Cu_B site causes the rapid electron transfer in the oxygen reaction.

In this paper this property of the peroxide reaction is further investigated by applying higher peroxide concentrations. Furthermore, we report on the pH dependence and the temperature dependence of the oxidation of cytochrome *c* oxidase by hydrogen peroxide. Finally, the reaction was studied in ^2H_2O in order to establish which steps in the reaction mechanism of cytochrome *c* oxidase are associated with proton translocation.

A preliminary report of some of the results discussed in this paper was recently presented at the UNESCO International Workshop on Cytochrome systems at the University of Bari, Italy.

Materials and Methods

Bovine cytochrome *c* oxidase was isolated according to Refs. 32 and 33; cytochrome *c* was isolated according to Ref. 34. Reduced cytochrome *c* was obtained by incubating cytochrome *c* with ascorbate, followed by gel filtration in 25 mM Tris-acetate/1 mM EDTA (pH 7.8) on Sephadex G-50 Superfine (Pharmacia). Concentrations were determined using an absorbance coefficient (reduced-oxidised) at 550 nm of 21.1 $mM^{-1} \cdot cm^{-1}$ [35].

Unless otherwise indicated, cytochrome *c* oxidase was dissolved in 100 mM potassium phosphate (pH 7.4), 50 mM glucose and 1% Tween-80. The oxidase concentration was determined spectrophotometrically using an absorbance coefficient (reduced-oxidised) of 24.0 $mM^{-1} \cdot cm^{-1}$ at 605 nm [36]. All experiments were performed at 20 °C unless otherwise indicated.

H_2O_2 (Suprapur), ^2H_2O and glucose were from Merck (Darmstadt), sodium dithionite and sodium ascorbate were from British Drug Houses, and glucose oxidase (*Aspergillus niger*, grade I) was from Boehringer Mannheim GmbH.

The rapid-scan/stopped-flow measurements were carried out with a Union-Giken RA-401 spectrophotometer. Whenever possible, reaction rate constants were calculated by using the first-order fitting program this apparatus is equipped with. When the reaction of reduced cytochrome *c* oxidase with hydrogen peroxide was studied, one stopped-flow reservoir contained the cytochrome *c* oxidase solution in the presence of a small excess of sodium dithionite, whereas the other reservoir contained hydrogen peroxide. To convert the oxygen dissolved in the buffer into hydrogen peroxide, a small amount of glucose oxidase was present in this reservoir. This experimental method is described in Ref. 18, and was previously applied by Bickar et al. [19].

When the reaction of pulsed oxidised cytochrome *c* oxidase with H_2O_2 was studied, the conditions were identical to those described above, except that glucose oxidase was omitted. Under these conditions, after mixing the reduced enzyme will be rapidly reoxidised by the oxygen, present in the H_2O_2-containing reservoir, resulting in 100% formation of the pulsed enzyme, which will then, in turn, react with H_2O_2 [18]. When the reaction of H_2O_2 with resting oxidised cytochrome *c* oxidase was studied, the measuring conditions were the same as those of the pulsed-enzyme experiments, except that no sodium dithionite was added to the cytochrome *c* oxidase solution. When the reaction of reduced cytochrome *c* with oxidised cytochrome *c* oxidase was studied, one reservoir contained the reduced cytochrome *c* solution, while the other contained the cytochrome *c* oxidase solution.

The pH was varied by using buffer solutions of different pH, but with identical ionic strengths. The ionic strength was kept constant ($I = 272$ mM) by addition of varying amounts of potassium chloride. As buffering agents potassium phosphate (pH 6.0–8.8) and Tris-HCl (pH 8.0–9.5) were used. For the experiments carried out in the presence of ^2H_2O a correction was made to account for the difference in sensitivity of the electrode for protons and deuterons by adding a value of 0.4 to the pH meter readings for ^2H_2O. Also, in order to take into account the different dissociation constants for $^2H^+$ and H^+, experiments in ^2H_2O were compared with those in H_2O at a p^2H that was 0.5 higher than the pH.

One might wonder whether our results are in any way associated with the catalase activity which is present in most cytochrome *c* oxidase preparations. We already reported in Ref. 29 that in our preparation, which is of the modified Fowler type, the catalase activity is negligibly low, but since the peroxide concentrations used in this paper are considerably higher than those used in Ref. 29, we thought it be necessary to re-check the catalase activity in our preparation. The results confirmed the observations in Ref. 29, which means that the catalase activity (17 $M^{-1} \cdot s^{-1}$ with reference to the oxidase concentration) is more than an order of magnitude lower than the slowest reaction, observed in this paper (see Results, $k = 700$ $M^{-1} \cdot s^{-1}$). We can therefore rule out the possibility that any of the results presented here are due to an oxygen evolution catalysed by catalase. Since in this paper no experiments with oxygen are reported, it may be appropriate to recall that our reduced preparation is re-oxidised by oxygen within a few milliseconds [18]. Moreover, it has a high turnover number, varying from 300 to 500 s^{-1} under physiological conditions.

Results

When hydrogen peroxide is added to reduced cytochrome *c* oxidase the enzyme is oxidised. In a previous paper [18] we showed that at high peroxide concentrations the rate of cytochrome *a* oxidation, as measured at 428 nm and at 605 nm, was much slower than the rate of cytochrome a_3 oxidation, as measured at 436 nm. This is caused by the fact that the cytochrome *a* oxidation rate is limited by the rate of electron transfer from cytochrome *a* to cytochrome a_3. We now report the rate of cytochrome *a* oxidation at yet higher peroxide concentrations (up to 44 mM) (Fig. 1). At these concentrations – at which the cytochrome a_3 oxidation is too fast to measure with stopped-flow methods – the rate of cytochrome *a* oxidation did not approach a limiting value of 15–25 s^{-1}, as we presumed earlier, but exhibited a slow increase beyond this value. This continuous increase of the observed first-order rate constant with the hydrogen peroxide concentration could be fitted by a straight line, with a slope of 700 $M^{-1} \cdot s^{-1}$ and an intercept at zero H_2O_2 concentration of 16 s^{-1}.

In the same range of peroxide concentrations the rapid-scan spectra (not shown) exhibited the same order of spectral changes throughout the concentration range: cytochrome a_3 oxidation was followed by cytochrome *a* oxidation, after which the cytochrome $a_3^{3+} \cdot H_2O_2$ complex was formed. Even at the highest H_2O_2 concentrations applied, the peroxide complex formation did not precede cytochrome *a* oxidation. No new intermediates could be identified.

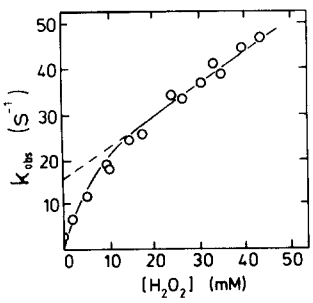


Fig. 1. Dependence of the observed rate constant for the oxidation of cytochrome *a* on the hydrogen peroxide concentration. The reaction was measured at a wavelength of 605 nm. The cytochrome *c* oxidase concentration was 2.3 μM. See Materials and Methods for experimental details.

The pH-dependence of the oxidation of cytochrome *a* and a_3 by H_2O_2 was measured in a pH range of 6.4–8.9 (Fig. 2). The rate of oxidation of cytochrome a_3 (Fig. 2A) was found to be independent of pH, whereas that of cytochrome *a* (Fig. 2B) decreased with increasing pH. This indicates that the rate of electron transfer from cytochrome *a* to cytochrome a_3 is slightly affected by protonation. It should be noted, however, that particularly below pH 7.4 this pH effect was not always present in each preparation. The pH-dependence of the peroxide-complex formation rate with oxidised cytochrome *c* oxidase was also measured (not shown). We observed that the complex-formation rate of H_2O_2 with pulsed oxidised cytochrome *c* oxidase as measured at 435 nm, at which wavelength the absorbance changes are monophasic [18], was independent of pH (pH 6.4–8.0). Complex formation of hydrogen peroxide with the enzyme as isolated (the resting enzyme) is multiphasic [18,20]. We observed that the first phase, which unlike the subsequent phase is dependent on the peroxide concentration, was also pH independent. However, the contribution of the first phase to the total absorbance change depended on pH: with increasing pH this fraction increased from 17% at pH 6.4 to 34% at pH 8.0.

The temperature dependence of cytochrome a_3 and cytochrome *a* oxidation by H_2O_2 was measured between 3 and 33°C. The Arrhenius plots (Fig. 3) could be fitted with straight lines, from which activation energies were calculated. The activation energies for cytochrome a_3 oxidation and cytochrome *a* oxidation were 23 and 45 $kJ \cdot mol^{-1}$, respectively. The temperature dependence of the reaction of H_2O_2 with oxidised pulse cytochrome *c* oxidase was also measured (not shown). The activation energy for this reaction was found to be 37 $kJ \cdot mol^{-1}$.

Finally, the effect of replacing H_2O by ^2H_2O as the solvent was investigated (Fig. 4). In ^2H_2O the rates of cytochrome a_3 oxidation by H_2O_2 (Fig. 4A) were lower by 15% in comparison with the rates in H_2O. For the cytochrome *a* oxidation rates in H_2O or ^2H_2O (Fig. 4B) the slopes of the linear parts of the curves are similar. The inter-

Fig. 2. The effect of pH on the rate of oxidation of cytochrome a_3 (Fig. 2A) and of cytochrome a (Fig. 2B) by hydrogen peroxide. The oxidation of cytochrome a_3 was measured at 436 nm, the oxidation of cytochrome a at 605 nm. The cytochrome c oxidase concentration was 2.3 μM. The oxidation of cytochrome a and a_3 was measured with 7.5 mM hydrogen peroxide. See Materials and Methods for experimental details.

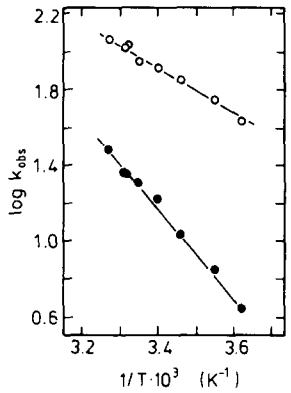


Fig. 3. The effect of temperature on the oxidation of cytochrome c oxidase by hydrogen peroxide. The oxidation of cytochrome a (closed circles) and a_3 (open circles) was measured at 605 nm and 436 nm, respectively. The reaction rates were measured with 2.3 μM cytochrome c oxidase and 7.5 mM hydrogen peroxide. See Materials and Methods for experimental details.

cept, however, decreased with about 50% on deuteration. The curves shown here were measured at pH 7.4 (p^2H 7.9). Similar ^2H_2O effects could also be measured at pH 6.5 and pH 8.6 (not shown).

We also investigated the effect of ionic strength on the reactions studied in this paper. The ionic strength was varied between 25 mM and 425 mM. The results showed that neither the oxidation of cytochrome a_3 and of cytochrome a by H_2O_2 nor the reaction of H_2O_2 with oxidised cytochrome c oxidase was in any way influenced by ionic strength.

The experiments with ^2H_2O described thus far were designed to yield information about involve-

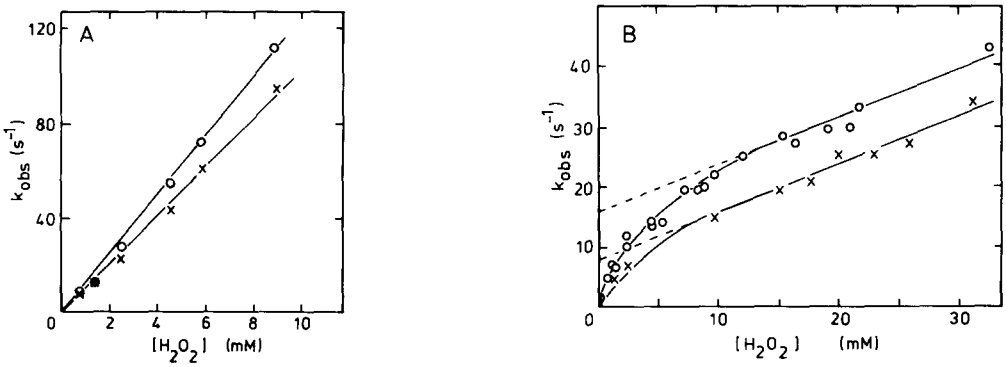


Fig. 4. The effect of ^2H_2O on the oxidation rate of cytochrome c oxidase by hydrogen peroxide. The observed rate constants for cytochrome a_3 oxidation (Fig. 4A) and cytochrome a oxidation (Fig. 4B) are shown as a function of the peroxide concentration, both in H_2O (○) and in ^2H_2O (×). The cytochrome c oxidase concentration was 2.3 μM. The experiments were performed at pH 7.4 and p^2H 7.9.

TABLE I

EFFECT OF ^2H_2O ON THE APPARENT RATE CONSTANTS OF THE REACTION BETWEEN REDUCED CYTOCHROME *c* AND OXIDIZED CYTOCHROME *c* OXIDASE

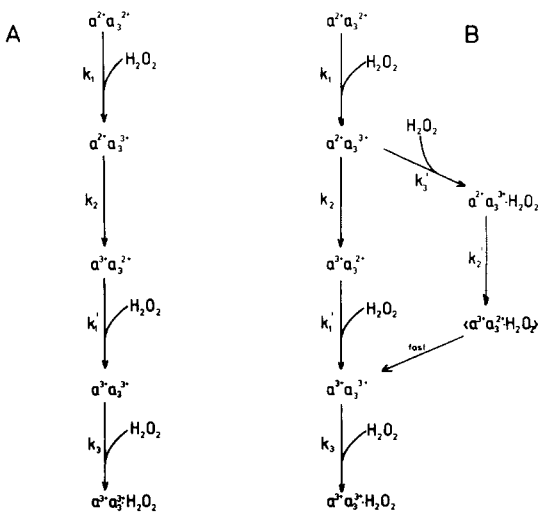
The reactions were measured in a buffer of phosphate/KCl with 1% Tween-80 (I = 272 mM); the temperature was 10 °C, except for the experiment at pH 7.2 (p^2H 7.7) which was performed at 25 °C. Cytochrome *c* concentrations were varied between 3.0 and 9.6 μM. The cytochrome *c* oxidase concentration was 1.0 μM. The reaction was measured at 444 nm. For each pH, the observed first-order rate constants were plotted as a function of the cytochrome *c* concentration. These plots were fitted with straight lines. The slopes of these lines are presented in the table as $k_{1_{app}}$, the intercepts on the *Y*-axis as $k_{-1_{app}}$.

pH	p^2H	$k_{1_{app}}$ ($\mu M^{-1} \cdot s^{-1}$)			$k_{-1_{app}}$ (s^{-1})		
		k_{H_2O}	$k_{^2H_2O}$	$\frac{k_{^2H_2O}}{k_{H_2O}}$	$k_{^2H_2O}$	$k_{^2H_2O}$	$\frac{k_{^2H_2O}}{k_{H_2O}}$
5.9	6.5	0.5	0.3	0.6	3.5	2.0	0.6
7.0	7.4	1.2	1.4	1.2	2.2	2.8	1.3
7.2	7.7	5.3	5.8	1.1	8.0	5.8	0.7
8.0	8.5	2.6	2.9	1.1	11.2	3.8	0.4

ment of protons in reactions at the oxygen-binding site and in the internal electron transfer from cytochrome *c* and Cu_A to cytochrome a_3 and Cu_B. However, we also wanted to investigate whether protons are taking part in the reaction at the cytochrome *c* binding site. Therefore we studied the effect of ^2H_2O on the pre-steady-state reaction of reduced cytochrome *c* with oxidised cytochrome *c* oxidase. At several pH values (and matching p^2H values) the observed first-order rate constant of this reaction was measured as a function of the cytochrome *c* concentration. From the results of these measurements apparent association and dissociation rate constants were derived which are present in Table I. The results indicate that the cytochrome *c* association rate constant is not affected very much by ^2H_2O, while the effects of ^2H_2O on $k_{-1_{app}}$ are rather ambiguous.

Discussion

The reaction of reduced cytochrome *c* oxidase with hydrogen peroxide was previously reported by us in two papers [18,29]. The results were explained by a linear reaction sequence as represented in Scheme IA. According to this scheme, the reaction consists of the oxidation of reduced cytochrome a_3 (and Cu_B) by a hydrogen peroxide molecule ($k_1 = 2 \cdot 10^4\ M^{-1} \cdot s^{-1}$) and the internal transfer of two electrons from cytochrome *a* and Cu_A to cytochrome a_3 and Cu_B ($k_2 \approx 20\ s^{-1}$), followed by the oxidation of the a_3-Cu_B site by a second peroxide molecule ($k_1' = 2 \cdot 10^4\ M^{-1} \cdot s^{-1}$). Finally, oxidised cytochrome a_3 will form a complex with yet a peroxide molecule in a comparatively slow reaction ($k_3 = 700\ M^{-1} \cdot s^{-1}$). This



Scheme I. The reaction sequence which describes the events that occur after addition of hydrogen peroxide to reduced cytochrome *c* oxidase. Scheme IA shows the linear reaction sequence that adequately describes the events, provided the peroxide concentration is kept low (less than 2 mM). Scheme IB depicts the reaction sequence taking into account the possibility of $a_3^{3+} \cdot H_2O_2$ complex formation in the enzyme state $a^{2+}a_3^{3+}$. The copper atoms have been left out for the sake of simplicity.

reaction scheme will suffice as long as the peroxide concentration is low. However, when high peroxide concentrations are applied, and the enzyme is in the $a^{2+}a_3^{3+}$ state, the complexation reaction of H_2O_2 with this enzyme state is fast enough to compete with the internal electron transfer from a^{2+} to a_3^{3+}. This obliges us to introduce a branch in the reaction sequence as shown in Scheme IB.

In our previous paper [18] we payed much attention to the surprising result that with H_2O_2 as the electron acceptor the electron transfer from cytochrome *a* and Cu_A to the a_3-Cu_B site was slow, and several explanations were suggested. The one preferred by us was that it is the formation of the peroxo-intermediate, which is formed in the oxygen reaction but not in the peroxide reaction, that causes the very fast electron transfer of cytochrome *a* to cytochrome a_3 (k in the order of 10^3 s^{-1}). Other explanations, however, could at that moment not be ruled out (see Ref. 18). The results reported here for high H_2O_2 concentrations render new possibilities to test our hypothesis.

If our hypothesis is wrong, the formation of a peroxide complex will not affect the internal electron-transfer rate and k_2' will be equal to k_2. It is evident that in that case, for high peroxide concentrations the rate equation will take on the form:

$$v = k_2[a^{2+}a_3^{3+}] + k_2[a^{2+}a_3^{3+} \cdot H_2O_2] = k_2[a^{2+}]_{total}$$

Thus cytochrome *a* oxidation could, for high peroxide concentrations, be approximated as a first-order reaction with an observed rate constant k_2. In other words, the introduction of the branch would have no effect at all on the oxidation of cytochrome *a*, and we would expect to find the same dependence of k_{obs} on $[H_2O_2]$ that we derived for the unbranched Scheme IA, i.e., k_{obs} approximates k_2 for high peroxide concentrations. For low peroxide concentrations the cytochrome *a* oxidation rate is governed by the oxidation of cytochrome a_3 by H_2O_2 (k_{obs} is about $\frac{1}{2}$ $k_1 \cdot [H_2O_2]$, as explained in Ref. 18). The expected curve is shown in Fig. 5 (curve a). We would also expect to observe $a_3^{3+} \cdot H_2O_2$ complex formation prior to cytochrome *a* oxidation in rapid-scan spectra for high peroxide concentrations, as the

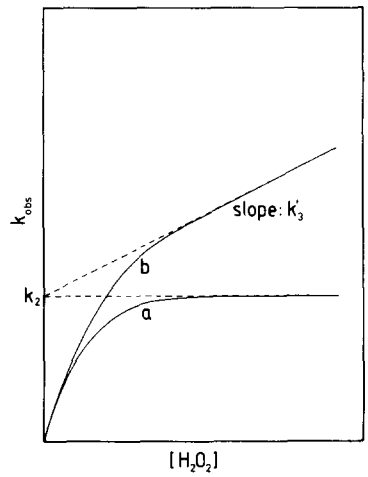


Fig. 5. Theoretical relationship between the observed rate of cytochrome *a* oxidation and the concentration of hydrogen peroxide as derived from the reaction sequence represented in Scheme IB. Curve a is found if $k_2' = k_2$. Curve b is found if $k_2' \gg k_2$ (the slope of the linear part of curve b will yield the rate constant k_3').

intermediate $a^{2+}a_3^{3+} \cdot H_2O_2$ will accumulate when $k_3' \cdot [H_2O_2]$ becomes higher than k_2'.

If our hypothesis is correct, the formation of the $a_3^{3+} \cdot H_2O_2$ complex in the branch will cause a large increase in the internal electron-transfer rate and k_2' will be in the order of 10^3 s^{-1}. It can be deduced that, in this case, for high peroxide concentrations the rate equation for cytochrome *a* oxidation will be:

$$v = (k_2 + k_3' \cdot [H_2O_2]) \cdot [a^{2+}a_3^{3+}]$$

$$= (k_2 + k_3' \cdot [H_2O_2]) \cdot [a^{2+}]_{total}$$

This also represents a first-order reaction, but now the observed rate constant k_{obs} is dependent on the peroxide concentration: $k_{obs} = k_2 + k_3' \cdot [H_2O_2]$. A plot of k_{obs} as a function of $[H_2O_2]$ would then, for high peroxide concentrations, display a linear relationship with a slope that equals k_3' and an intercept of k_2. The expected curve is shown in Fig. 5 (curve b). If we assume that the redox states of cytochrome *a* and Cu_A have no effect on the complex formation rate, as we showed earlier for the reaction of the reduced enzyme with hydrogen peroxide [29], k_3' will be 700 $M^{-1} \cdot s^{-1}$

and so will the slope of the curve. In this case cytochrome *a* oxidation will always precede $a_3^{3+} \cdot H_2O_2$ complex formation.

From the results obtained by us we fell we can now firmly conclude that our original hypothesis was correct. Cytochrome *a* oxidation always preceded $a_3^{3+} \cdot H_2O_2$ complex formation, and the plot of k_{obs} vs. $[H_2O_2]$ exhibited a linear relationship for high peroxide concentrations (slope 700 $M^{-1} \cdot s^{-1}$). The intercept gave a value of 16 s^{-1}, which in our view represents the true value of the internal electron-transfer rate in the absence of the peroxo-intermediate. The rate of internal electron-transfer in the presence of the peroxide complex cannot be directly deduced from these experiments, but it must at least be one order of magnitude faster than peroxide-complex formation at the highest peroxide concentration applied (30 s^{-1} at a concentration of 44 mM), in order to explain the fact that at concentration of H_2O_2 cytochrome *a* oxidation still precedes peroxide complex formation. This sets a lower limit for that rate of 300 s^{-1}, which brings it close to the value reported for the oxygen reaction (700 s^{-1}) [30].

The reaction rate of hydrogen peroxide with reduced cytochrome a_3 was independent of pH. The reaction involved is the association of hydrogen peroxide to the reduced oxygen-binding site, since the rate is linearly dependent on the peroxide concentration, as we showed previously [18,29]. The pH-independence of this reaction shows that no acid-base groups on the enzyme are involved and that it is the H_2O_2 molecule, and not a (de)protonated form, that reacts with cytochrome a_3^{2+}. By its independence of pH the peroxide reaction resembles the oxygen reaction which is also independent of pH [37,38].

The similarity between O_2 and H_2O_2 reactions is further demonstrated by the pH-dependence of the cytochrome *a* oxidation rate. For the oxygen reaction a three-fold decrease in the internal electron-transfer rate was observed when the pH was raised from pH 7 to pH 9 [37]. For the peroxide reaction we also found a gradual decrease (2.5-fold) in the rate of cytochrome *a* oxidation upon increasing the pH. When we studied the reaction of H_2O_2 with oxidised pulsed cytochrome *c* oxidase, we found that the rate of complex formation was independent of pH. Since this rate was linearly dependent upon the peroxide concentration, this means that the association of hydrogen peroxide to oxidised pulsed cytochrome *c* oxidase is independent of pH. The multiphasic complex formation of hydrogen peroxide with the resting enzyme was previously [18,20] explained by the inhomogeneity of the enzyme as isolated. Our observation that the fraction of enzyme that reacted with hydrogen peroxide in the first, $[H_2O_2]$-dependent phase was a function of the pH, confirms this. We found that the fast-reacting enzyme fraction increased upon increasing pH. This agrees with the pH dependence of hydrogen peroxide binding reported by Bickar et al. [19], and with the pH dependence of the composition of oxidised enzyme forms [11,39–41].

The value determined for the activation energy of the oxidation of cytochrome a_3^{2+} by H_2O_2 (23 $kJ \cdot mol^{-1}$) was rather low for an enzyme-catalysed reaction. A similar low value was also reported for the reaction with oxygen (16–17 $kJ \cdot mol^{-1}$) [38,42]. The effect of temperature on the rate of internal electron transfer from *a* to a_3, as obtained in the oxidation of cytochrome *c* oxidase by H_2O_2 (activation energy of 45 $kJ \cdot mol^{-1}$), was nearly the same as that found by Orii et al. [42] using O_2 as the oxidant (49.8 $kJ \cdot mol^{-1}$). These very similar values suggest that for both reactions the transfer of electrons proceeds along the same pathway. The great difference in reaction rates must be explained by a difference in activation entropy.

The ability of cytochrome *c* oxidase to translocate protons across the mitochondrial inner membrane is of obvious interest and for this reason experiments in ^2H_2O have been performed [43–45]. Thornström and Malmström [46] reported that cytochrome *c* oxidase exhibited a 2- to 3-fold decrease in activity with ^2H_2O as the solvent, in line with Ref. 45, as discussed in Ref. 46. This suggests that a rate-limiting step probably involves proton translocation. In order to find out which steps in the mechanisms are affected, the effect of ^2H_2O was studied on the cytochrome *c* reaction, the internal electron transfer and the peroxide reaction, respectively. The H_2O_2-association rate constant decreased by about 15% in ^2H_2O. The nature of this effect is not clear, but the effects seem too small to be caused by direct

involvement of protons. It should be borne in mind that in ^2H_2O hydrogen peroxide is deuterated as well. The effect observed by us could be due to this, even though the protons in H_2O_2 are not expected to participate directly in the reaction. The effect of ^2H_2O on the internal electron transfer is much more striking. In this case, the reaction rate decreased two-fold. This suggests that the internal electron transfer probably involves proton translocation, in line with the hypothesis of Malmström et al. [47,48]. Concerning the cytochrome *c* reaction we have to conclude that the second-order association rate constant for cytochrome *c*, which is identical to $k_{1_{app}}$, is little or not affected by ^2H_2O. It would be more important to know whether there is an effect of ^2H_2O on the cytochrome *c* dissociation rate. However, the results we obtained for $k_{-1_{app}}$ are too ambiguous to allow positive conclusions; moreover, as the association of cytochrome *c* is followed by very fast electron redistributions between cytochrome *c*, cytochrome *a* and Cu_A, $k_{-1_{app}}$ is not only a function of the dissociation rate constant, but also depends on the redox potentials of the redox centres.

In summary, we reached the following conclusions. The formation of a complex of oxidised cytochrome a_3 with hydrogen peroxide strongly enhances the rate of electron transfer from cytochrome *a* to cytochrome a_3. This effect explains the difference in the rate of oxidation of cytochrome *a* by H_2O_2 and by O_2. The mechanism that underlies this rate enhancement cannot be deduced from our experiments. It might either be caused by an increase in the redox potential of the oxygen-binding site or by a conformational change. In this respect the studies of Boelens et al. [49] are of interest. They showed that upon photodissociation of mixed-valence carboxy-cytochrome *c* oxidase a fast electron transfer from cytochrome a_3 to Cu_A occurred. Although this seems to indicate that redox potential differences control the rate of electron transfer between cytochrome *a* and cytochrome a_3, it may very well prove to be that in this case the enzyme is in a particular conformational state in which electron transfer is rapid. The pH and temperature dependence of the rate of oxidation of cytochrome *a* and a_3 by H_2O_2 show that, despite the large difference in the values of the rate constants of the O_2 and H_2O_2 reactions, these reactions are similar in many respects. The observations in ^2H_2O suggest that the internal electron transfer might be associated with proton translocation. It would be most interesting to establish whether the internal electron transfer in cytochrome *c* oxidase exhibits a similar effect of ^2H_2O when oxygen is the final electron acceptor.

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