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The oxidation of cytochrome *c* oxidase by hydrogen peroxide *

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The reaction of H_2O_2 with mixed-valence and fully reduced cytochrome *c* oxidase was investigated by photolysis of fully reduced and mixed-valence carboxy-cytochrome *c* oxidase in the presence of H_2O_2 under anaerobic conditions. The results showed that H_2O_2 reacted rapidly ($k = (2.5–3.1) \cdot 10^4$ $M^{-1} \cdot s^{-1}$) with both enzyme species. With the mixed-valence enzyme, the fully oxidised enzyme was reformed. On the time-scale of our experiments, no spectroscopically detectable intermediate was observed. This demonstrates that mixed-valence cytochrome *c* oxidase is able to use H_2O_2 as a two-electron acceptor, suggesting that cytochrome *c* oxidase may under suitable conditions act as a peroxidase. Upon reaction of H_2O_2 with the fully reduced enzyme, cytochrome *a* was oxidised before cytochrome a_3. From this observation it was possible to estimate that the rate of electron transfer from cytochrome *a* to a_3 is about 0.5–5 s^{-1}.

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 metal centres as prosthetic groups: two haem *a* groups and two coppers (Cu_A and Cu_B) [1]. Two of these groups, the haem *a* group in cytochrome *a* and Cu_A are involved in accepting electrons from cytochrome *c*, whereas the other two, the haem *a* group in cytochrome a_3 and Cu_B, form a binuclear reaction site where oxygen binding and reduction takes place [2,3]. Emphasis has lately been laid upon the reaction of this binuclear site in oxidised cytochrome *c* oxidase with H_2O_2, which is known to form a stable complex with the enzyme [4–7]. The reason for this interest is that a peroxy compound is proposed to be an intermediate in the reaction of oxygen with reduced cytochrome *c* oxidase [5,8–17]. Until now, however, the reaction of H_2O_2 with reduced or mixed-valence cytochrome *c* oxidase has not received much attention, although its existence was reported many years ago [18]. It was demonstrated [4,5] that cytochrome *c* oxidase catalyses the peroxidative oxidation of ferrocytochrome *c* and the enzyme is apparently able to use hydrogen peroxide as an electron acceptor. This observation prompted us to study the reaction of fully reduced and mixed-valence enzyme with H_2O_2 in more detail.

Materials and Methods

Bovine cytochrome *c* oxidase was isolated according to Ref. 20; and cytochrome *c* was isolated according to Ref. 21. Anaerobiosis was accomplished by repeated cycles of evacuation and flushing with helium. Mixed-valence carboxy-cytochrome *c* oxidase was prepared by incubation of

* This paper is dedicated to the memory of Dr. J.H. Meijling, who designed and constructed an excellent spectrophotometer using the optics of an outdated Cary-14.

cytochrome *c* oxidase for several hours with CO at room temperature [22] under anaerobic conditions. Fully reduced carboxy-cytochrome *c* oxidase was prepared by addition of excess sodium ascorbate (5 mM) and a small amount of cytochrome *c* (60–600 nM) to cytochrome *c* oxidase in the absence of O_2, after which CO was added to the sample. Experiments were carried out in Thunberg cuvettes with two side bulbs, one of which contained ascorbate and cytochrome *c*, and the other contained H_2O_2. This side bulb was kept in melting ice.

H_2O_2 was from Merck (Suprapur), ascorbic acid was from British Drug Houses (Analar), helium was from Hoek Loos, and CO from Matheson Gas Products.

Absorption spectroscopy was carried out on a Cary-14 spectrophotometer extensively modified by Dr. J.H. Meijling to obtain digital rather than analog results. The kinetic difference spectra were obtained on a Hewlett Packard 8451 diode array spectrophotometer. With this instrument it is possible to obtain optical absorption spectra within 1 s. Photodissociation of the sample was achieved by cross-beam illumination with a 150 W xenon continuous lamp (Oriel) with appropriate optical filters in front of the xenon lamp and the photomultiplier or diode array (OG 570 (Schott) and BG 12 (Schott) and vice versa, plus a Calflex heat reflection filter immediately in front of the xenon lamp). This was done to prevent actinic light from falling onto the photomultiplier or diode array. Actinic light attenuation was obtained by placing a calibrated neutral density filter (Oriel) in the actinic light beam.

CO concentrations in solution were calculated according to Henry's Law with a solubility of CO in water of 1 mM at a pressure of 100 kPa [23].

Cytochrome *c* oxidase was dissolved in 100 mM potassium phosphate (pH 7.4) and 1% Tween-80 to a final concentration of 8–10 μM. Its concentration was determined spectrophotometrically using an absorption coefficient (red-ox) of 24.0 $mM^{-1} \cdot cm^{-1}$ at 605 nm [24].

Results

Experimental set-up and kinetic evaluation

Since H_2O_2 will react rapidly with mixed-valence or fully reduced enzyme [18] under anaerobic conditions, it is difficult to monitor this reaction in a direct way. However, in the presence of CO and in the dark, reduced cytochrome a_3 reacted only very slowly with H_2O_2 (not shown). This is due to the high affinity of CO for reduced cytochrome a_3 [25]. The cytochrome $a_3^{2+} \cdot CO$ complex is photodissociable and this property will enable us to study the reaction between cytochrome *c* oxidase and H_2O_2 by continuous illumination of the sample. By this procedure we were able to mix H_2O_2 with cytochrome *c* oxidase before starting the reaction.

We have to bear in mind, however, that upon continuous illumination photodissociation is incomplete. Under these conditions the reaction will proceed according to:

$$a_3^{2+} \cdot CO \underset{k_{on}}{\overset{k_{off}(light)}{\rightleftharpoons}} a_3^{2+} \underset{CO}{\overset{k_1}{\rightarrow}} a_3^{3+} \underset{H_2O_2}{}$$

Scheme I

where k_{on} and k_{off} are the association and dissociation rate constants of the equilibrium reaction between CO and cytochrome a_3, respectively, and k_1 is the second-order rate constant of the reaction between reduced cytochrome a_3 and H_2O_2. If $k_{on} + k_{off} \gg k_1$ the reaction can be described as a slow oxidation reaction of cytochrome a_3 coupled to a fast association-dissociation equilibrium between cytochrome a_3^{2+} and CO. For this model it can be shown that the reaction is first-order and that the measured rate constant k^* is equal to:

$$k^* = k_1[H_2O_2] \cdot f \tag{1}$$

where f is the dissociated fraction of the CO complex; f is given by the equation:

$$f = \frac{[a_3^{2+}]}{[a_3^{2+}] + [a_3^{2+} \cdot CO]} \tag{2}$$

This can be rewritten as:

$$f = \frac{1}{\frac{[CO]}{K_d} + 1} \tag{3}$$

in which K_d is the dissociation constant for the cytochrome $a_3^{2+} \cdot CO$ complex. This constant is a

function of the light intensity falling on the sample, and may vary from experiment to experiment. Therefore, the fraction dissociated (f) was determined each time by illuminating the sample before H_2O_2 was mixed with the enzyme. The light-induced absorption difference between 428 nm (trough) and 445 nm (peak) measured in this way was divided by the absorbance difference at these wavelengths of reduced enzyme minus that of the reduced enzyme ligated to CO.

Reaction of mixed-valence cytochrome c oxidase with H_2O_2

The oxidation of mixed-valence CO cytochrome c oxidase by H_2O_2 during illumination was followed in time by continuously measuring the absorbance at 415 nm. The absorbance changes at this and at other wavelengths were always first-order in the $[H_2O_2]$ range investigated. Fig. 1 shows that the measured rate constants depend linearly on the hydrogen peroxide concentration. Using Eqn. 1, a second-order rate constant of $k_1 = 2.5 \cdot 10^4\ M^{-1} \cdot s^{-1}$ could be determined.

In order to ensure that Eqn. 1 is indeed obeyed, the effect of the concentration of CO on the oxidation rate was also investigated. At all CO concentrations used, ranging from 130 μM to 790 μM, and with and without illumination, absorbance changes were first order and the measured rate constants were linearly dependent on the H_2O_2 concentration. Table I shows that the values of k_1, obtained at various CO concentrations and calculated by using Eqn. 1, are equal.

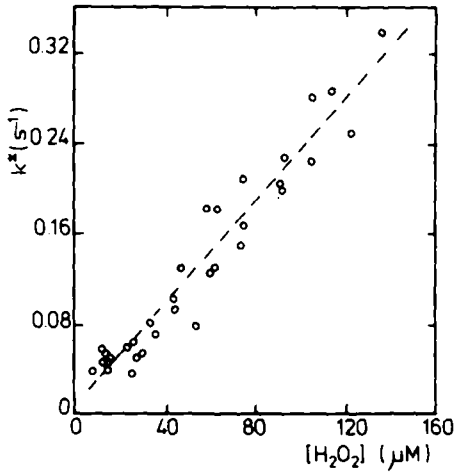


Fig. 1. Dependence of the oxidation rate of mixed-valence carboxycytochrome c oxidase on H_2O_2 concentrations. The reaction was started and maintained by continuous illumination of the sample. The oxidation of cytochrome a_3 was followed at 415 nm. Conditions: 8 μM cytochrome c oxidase, 100 mM potassium phosphate (pH 7.4), 1% Tween-80 and 790 μM CO. Dissociated fraction f, 0.092.

TABLE I

DETERMINATION OF k_1 FOR MIXED-VALENCE CYTOCHROME c OXIDASE AT VARIOUS CO CONCENTRATIONS

k^* is the measured first-order rate constant for the oxidation of carboxycytochrome c oxidase by H_2O_2, and equals $k_1 \cdot [H_2O_2] \cdot f$ (cf. Eqn. 1).

[CO] (μM)	Fraction	$k^*/[H_2O_2]$ ($10^3\ M^{-1} \cdot s^{-1}$)	k_1 ($10^4\ M^{-1} \cdot s^{-1}$)
789	0.076	1.8	2.4
395	0.141	3.5	2.5
263	0.198	4.7	2.4
132	0.330	8.2	2.5

It has been documented [18], that cytochrome c oxidase preparations show catalase activity. In order to ensure that the oxygen produced in this reaction did not interfere with our measurements, the rate of oxygen production was determined polarographically. Indeed, it was found that oxygen evolved when cytochrome c oxidase was added to H_2O_2. In line with the results reported in Ref. 18 the activity was low: under our conditions (10 μM cytochrome c oxidase) the rate constant of this reaction was 60 $M^{-1} \cdot s^{-1}$. This value is lower than the rate of oxidation by a factor of 400.

The reaction of fully reduced cytochrome c oxidase with H_2O_2

The same kind of experiments as reported above were carried out with fully reduced carboxy-cytochrome c oxidase. However, the absorbance changes upon illumination were not strictly first-order, the exact shape depended on the wavelength at which the absorbance was measured. This deviation from linearity was not caused by rereduction of cytochrome c oxidase by cytochrome c and ascorbate, since the rate of this reaction is very low ($t_{1/2} = 2$ min) due to the low concentration of cytochrome c.

Fig. 2 shows the absorbance changes caused by

addition of 30 μM H_2O_2 to the reduced CO complex and upon subsequent illumination at 432 nm. At this wavelength the absorbance changes caused by oxidation of cytochrome *a* and a_3 differ in sign according to Vanneste [26]. Oxidation of cytochrome *a* gives rise to an absorbance increase, while oxidation of cytochrome a_3 will lead to an absorbance decrease. Fig. 2 clearly shows that cytochrome *a* is oxidised before cytochrome a_3. This indicates that upon oxidation of cytochrome a_3 this redox component is reduced rapidly by cytochrome *a*. This requires, however, that internal electron transfer between cytochrome *a* and cytochrome a_3 is faster than oxidation of cytochrome a_3 by H_2O_2.

Fig. 3 shows the kinetic difference spectra obtained after photodissociation of fully reduced carboxy-cytochrome *c* oxidase in the presence of H_2O_2. It is clear from the shifting isosbestic point that two phases can be distinguished in the region 425–434 nm. In line with Fig. 2, one phase gives rise to an absorbance increase corresponding to oxidation of cytochrome *a*, while the absorbance decrease in the same wavelength region corresponds to oxidation of cytochrome a_3.

Fig. 4 shows the absorbance changes at 432 nm, but after addition of a much higher concentration of H_2O_2 (200 μM) than that used in Fig. 2. Fig. 4 differs from Fig. 2 in several aspects. In the first place there is a considerable absorbance increase

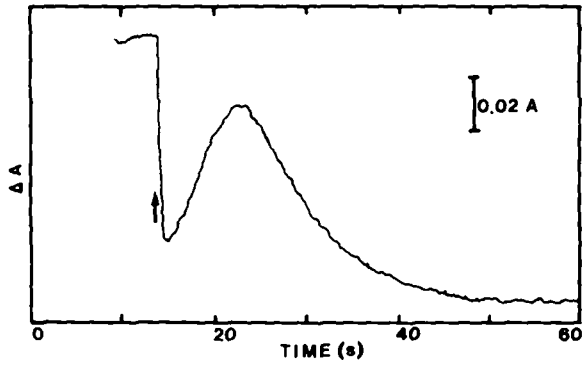


Fig. 2. Absorption changes at 432 nm of fully reduced carboxycytochrome *c* oxidase induced by 30 μM H_2O_2 and continuous illumination. At $t = 0$, H_2O_2 was added to the sample; the arrow indicates the time at which the continuous illumination was turned on. Conditions: 8 μM cytochrome *c* oxidase, 100 mM potassium phosphate (pH 7.4), 1% Tween-80 and 66 μM CO.

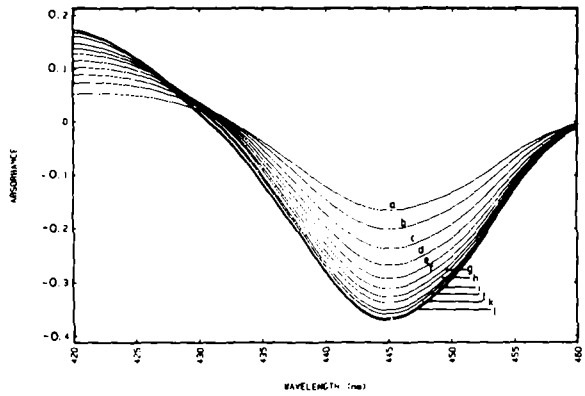


Fig. 3. Kinetic difference spectra of the oxidation of fully reduced cytochrome *c* oxidase by H_2O_2. Plotted is the absorbance difference induced by 50 μM H_2O_2 with respect to the absorbance spectrum of illuminated carboxy-cytochrome *c* oxidase before addition of H_2O_2. Conditions: 4.2 μM cytochrome *c* oxidase, 100 mM potassium phosphate (pH 7.4), 1% Tween-80 and 130 μM CO. (a) 1 s; (b) 2 s; (c) 3 s; (d) 4 s; (e) 5 s; (f) 6 s; (g) 7 s; (h) 9 s; (i) 11 s; (j) 20 s; (k) 30 s.

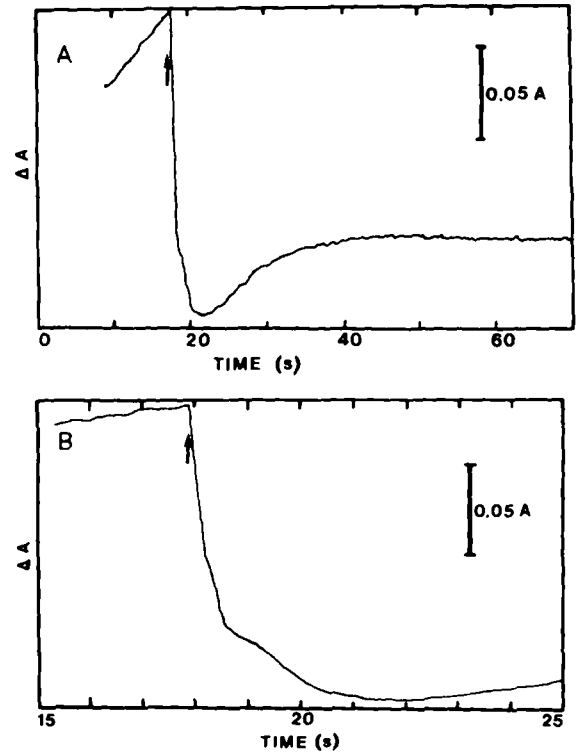


Fig. 4. Absorption changes at 432 nm of fully reduced carboxycytochrome *c* oxidase induced by 200 μM H_2O_2 and continuous illumination. Conditions as in Fig. 2, except for the higher H_2O_2 concentration. (A) Complete sequence of absorption changes; (B) part of (A) on an expanded scale.

in the dark, caused by oxidation of cytochrome *a*; secondly, the instantaneous absorption decrease upon illumination due to dissociation of CO is followed by a slower decrease due to oxidation of cytochrome a_3. The absorbance increase that was evident in Fig. 2 here only shows up as a small plateau around $t = 19$ s, indicating that in this case the internal electron-transfer rate from cytochrome *a* to cytochrome a_3 is of the same order of magnitude as the oxidation rate of cytochrome a_3. From Fig. 4 it can be estimated that the rate lies between 0.5 and 5 s^{-1}. This value is in agreement with those reported previously by others [27,28].

At these higher H_2O_2 concentrations also a slow absorbance change was observed that starts at 22 s (Fig. 4B). These changes are ascribed to formation of a stable cytochrome *c* oxidase-H_2O_2 complex [5,6]. Under the conditions of Fig. 2, internal electron transfer between cytochrome *a* and a_3 was faster than the oxidation of cytochrome a_3 by H_2O_2. This means that Eqn. 1 is no longer valid, since in scheme I electron transfer between cytochrome *a* and a_3 is not taken into account. It is possible to measure oxidation of cytochrome *a* to obtain the value of k_1, provided the oxidation rate is much slower than the internal electron transfer rate (cf. Discussion). Experimentally the oxidation rate can be slowed down by decreasing the intensity of the photodissociating light by a factor of 10 and by increasing the CO concentration to 1 mM. In order to obtain a measurable rate constant, the H_2O_2 concentration was increased to appreciably higher concentrations than used in the experiment of Fig. 1. In addition, the oxidation of cytochrome *a* was measured at 421.5 nm in order to avoid spectral interference with reactions involving cytochrome a_3.

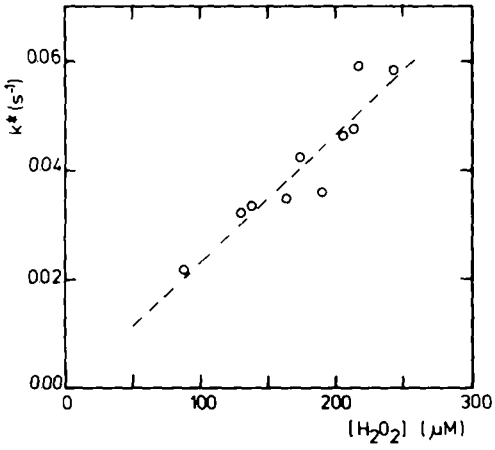


Fig. 5. Dependence of the oxidation rate of fully reduced carboxycytochrome *c* oxidase on H_2O_2 concentration. The reaction was started and maintained by continuous illumination of the sample. The reaction was followed at 421.5 nm. Conditions: 8 μM cytochrome *c* oxidase, 100 mM potassium phosphate (pH 7.4), 1% Tween-80 and 1 mM CO. Dissociated fraction *f*, 0.0071.

The resulting plot of measured rate constant as a function of the H_2O_2 concentration is shown in Fig. 5. At concentrations of H_2O_2 lower than 300 μM the plot is linear and from this a second-order rate constant of $3.1 \cdot 10^4$ $M^{-1} \cdot s^{-1}$ for the reaction between H_2O_2 and reduced enzyme could be calculated.

Discussion

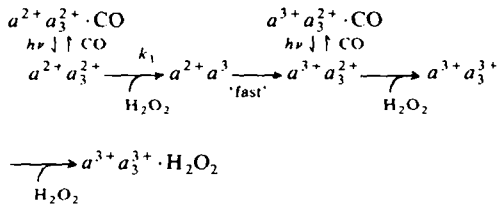
As far as the mixed-valence cytochrome *c* oxidase CO complex is concerned, the observations that the oxidation by H_2O_2 is strictly first-order in enzyme and that the reaction rate as a function of the concentration of H_2O_2 (Fig. 1) is linear, leave little doubt as to the validity of the simple Scheme I.

One may ask whether the electron redistributions which occur after photodissociation of the mixed-valence enzyme do not interfere with our measurements. It has been shown [29] that approximately 12% of cytochrome *a* and 7% of Cu_B becomes reduced upon illumination. The effect of this on the dissociated fraction as calculated by Eqn. 2 is negligible under our conditions (70 kPa, CO). Also, as determined experimentally, the fraction is not affected by the spectral differences observed between the light-induced difference spectra in mixed-valence and fully reduced carboxy-cytochrome *c* oxidase [30]. As shown in Fig. 3 of Ref. 30, the peak (445 nm)-to-trough (428 nm) absorbance difference is nearly the same for both species.

For the fully reduced enzyme, the events are more complicated. As shown in Figs. 2–4, after photodissociation of the cytochrome a_3^{2+}-CO complex, oxidation of cytochrome *a* is observed first, followed by oxidation of cytochrome a_3. This suggests an internal electron transfer from reduced

cytochrome a upon oxidation of cytochrome a_3 by H_2O_2. After oxidation of the enzyme to a partially reduced state this enzyme species reacts again with H_2O_2 to the fully oxidised enzyme. At high concentrations of H_2O_2 the fully oxidised enzyme forms a stable complex with H_2O_2 as described in Refs. 5 and 6.

Scheme II summarises the sequence of events.



Scheme II

Because of the internal electron transfer between the two cytochromes, evaluation of the second-order rate constant k_1 posed some problems. In principle it is possible to increase the rate of oxidation of cytochrome a_3 by H_2O_2 to a value above that of the rate of internal electron transfer merely by increasing the H_2O_2 concentration. However, under these conditions the rate becomes too fast to be observed experimentally. Therefore, another approach was used: experimental conditions were chosen which enabled us to measure oxidation of cytochrome a rather than of cytochrome a_3. For this purpose the oxidation rate of $a^{2+}a_3^{2+}$ to $a^{2+}a_3^{3+}$ was made rate-limiting. This can be done by using high concentrations of CO (1 mM) and a low intensity of dissociating light. The subsequent intramolecular electron transfer from cytochrome a to a_3 is then much faster than the rate of oxidation of cytochrome a_3 by H_2O_2. Thus, the measured oxidation rate of cytochrome a will be equal to the oxidation rate of cytochrome a_3 by H_2O_2.

Since under these conditions the concentration of the intermediate a_3^{2+} is low throughout the experiment, the only reactions that might interfere with the spectral measurements of the oxidation of cytochrome a are the conversion of $a_3^{2+}\cdot CO$ to a_3^{3+} and complex formation of a_3^{3+} with H_2O_2 to $a_3^{3+}\cdot H_2O_2$. The former reaction has an isosbestic point at 421.5 nm and the latter at 422.3 nm. Thus, by measuring the oxidation of cytochrome a at 421.5 nm, spectral interference by these reactions will be minimal.

The second-order rate constant k_1 calculated in this way was $3.1 \cdot 10^4\ M^{-1} \cdot s^{-1}$. The rate constant could also be obtained in another way and at other wavelengths. Assuming that after initiation of the reaction only cytochrome a is oxidised (cf. Fig. 2), it is possible to calculate the second-order rate constant from measurements of the initial rate of the absorbance change. This requires knowledge of the relative contribution of cytochrome a to the absorbance difference spectrum reduced minus oxidised. At 415 and 600 nm these contributions are 0.45 and 0.8, respectively, [26] and from these data values were obtained of $2.7 \cdot 10^4$ and $2.8 \cdot 10^4$ $M^{-1} \cdot s^{-1}$, in good agreement with the value calculated from Fig. 5.

The rate of the internal electron-transfer reaction from cytochrome a to a_3 was found to be in the order of 0.5–5 s^{-1}. This value is very similar to that calculated from the appearance of the photosensitivity of the cytochrome $a_3 \cdot CO$ compound after mixing the enzyme with cytochrome c and CO, as reported by Gibson et al. [27]. Thus, under our conditions the transfer of electrons from cytochrome a^{2+} to a_3^{3+} is a slow process compared to the normal turnover of the enzyme. Wilson et al. [28] in their two-state model also arrived at a rate constant for the electron transfer of about 1 s^{-1} for the resting oxidase and about 5 s^{-1} for the pulsed oxidase.

In Schemes I and II we have totally disregarded the role of the copper ions. However, when cytochrome a is being oxidised, Cu_A will probably be oxidised as well. This could seriously complicate Scheme II. It is, however, very likely that H_2O_2 takes up two electrons upon reaction with cytochrome c oxidase and under our measuring conditions the internal electron transfer reactions are fast in comparison to cytochrome a_3 oxidation. Because of this, cytochrome a oxidation will still be first-order and its rate will still be equal to the oxidation rate of cytochrome a_3 by H_2O_2.

Our experiment with the mixed-valence enzyme demonstrates that this species, in which both Cu_B and cytochrome a_3 are reduced [32,33], is able to use H_2O_2 as an electron acceptor. Since on the time-scale of our experiments no intermediate accumulates we suggest that both electrons on the

binuclear site in the enzyme are transferred simultaneously to the H_2O_2 molecule. The observation that partially and fully reduced cytochrome *c* oxidase are able to use H_2O_2 as an electron acceptor is in agreement with the data of Orii [4]. He showed that cytochrome *c* oxidase catalyses the peroxidative oxidation of ferrocytochrome *c* under anaerobic conditions. According to his data cytochrome *c* oxidase under air acted simultaneously as oxidase and peroxidase. This is surprising if one considers that the second-order rate constant for the reaction with H_2O_2 and with O_2 was found by us to be $2.5 \cdot 10^4$ and $1 \cdot 10^8$ $M^{-1} \cdot s^{-1}$, respectively [2]. The large difference in the value of these rate constants shows that the enzyme is extremely specific in its substrate reaction.

Acknowledgements

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