Biochimica et Biophysica Acta 852 (1986) 81–92
Elsevier

BBA 42126

Kinetic investigations of the reactions of cytochrome *c* oxidase with hydrogen peroxide

A.C.F. Gorren, H. Dekker and R. Wever

Laboratory of Biochemistry, University of Amsterdam, P.O. Box 20151, 1000 HD Amsterdam (The Netherlands)

(Received 7 February 1986)
(Revised manuscript received 6 June 1986)

Key words: Hydrogen peroxide; Cytochrome *c* oxidase; Electron transfer; Complex formation; Enzyme conformation

The reaction of H_2O_2 with reduced cytochrome *c* oxidase was investigated with rapid-scan/stopped-flow techniques. The results show that the oxidation rate of cytochrome a_3 was dependent upon the peroxide concentration ($k = 2 \cdot 10^4$ $M^{-1} \cdot s^{-1}$). Cytochrome *a* and Cu_A were oxidised with a maximal rate of approx. 20 s^{-1}, indicating that the rate of internal electron transfer was much slower with H_2O_2 as the electron acceptor than with O_2 ($k \geq 700$ s^{-1}). Although other explanations are possible, this result strongly suggests that in the catalytic cycle with oxygen as a substrate the internal electron-transfer rate is enhanced by the formation of a peroxo-intermediate at the cytochrome a_3-Cu_B site. It is shown that H_2O_2 took up two electrons per molecule. The reaction of H_2O_2 with oxidised cytochrome *c* oxidase was also studied. It is shown that pulsed oxidase readily reacted with H_2O_2 ($k \approx 700$ $M^{-1} \cdot s^{-1}$). Peroxide binding is followed by an H_2O_2-independent conformational change ($k = 0.9$ s^{-1}). Resting oxidase partially bound H_2O_2 with a rate similar to that of pulsed oxidase; after H_2O_2 binding the resting enzyme was converted into the pulsed conformation in a peroxide-independent step ($k = 0.2$ s^{-1}). Within 5 min, 55% of the resting enzyme reacted in a slower process. We conclude from the results that oxygenated cytochrome *c* oxidase probably is an enzyme-peroxide complex.

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

As a peroxy compound is proposed to be an intermediate in the reaction of oxygen with reduced cytochrome *c* oxidase [4–8], the reaction of oxidised cytochrome *c* oxidase with H_2O_2, which is known to form a stable complex with the enzyme, has been studied to some extent [9–12]. H_2O_2 also reacts with reduced cytochrome *c* oxidase [13], and in the presence of cytochrome *c* oxidase it is able to oxidise cytochrome *c* [9,10]. Those studies suggested that H_2O_2 can accept electrons from reduced cytochrome *c* oxidase, which was also indirectly shown by Brunori et al.

Correspondence address: Laboratory of Biochemistry, University of Amsterdam, P.O. Box 20151, 1000 HD Amsterdam, The Netherlands.

0005-2728/86/$03.50 © 1986 Elsevier Science Publishers B.V. (Biomedical Division)

[14] who studied the reduction of oxidised cytochrome *c* oxidase by sodium dithionite in the presence of hydrogen peroxide. In a previous study we investigated the oxidation of partially and fully reduced cytochrome *c* oxidase by H_2O_2 in some detail [15]. We showed that the reaction rate is linearly dependent on the peroxide concentration and that the reaction takes place at the oxygen-binding site. We also showed that the rate does not depend on the redox state of cytochrome *a* and Cu_A, and that the rate of electron transfer from cytochrome *a* to a_3 is rather low. However, the presence of carbon monoxide in those experiments was a complicating factor.

In this paper we extend our measurements to much higher peroxide concentrations and thus much higher reaction rates by using a rapid-scan/stopped-flow spectrophotometer, and we carried out these experiments in the absence of CO. Furthermore, we were able to compare directly the reactions of H_2O_2 and of O_2 with cytochrome *c* oxidase.

The oxidised enzyme is known to exist in different forms [16–20]. This diversity has caused considerable confusion and debate, leading to the definition of a number of enzyme forms, based on quite different criteria. The enzyme as isolated is usually referred to as the 'resting' form; this form itself is probably inhomogeneous, judging from its polyphasic reaction with many ligands and redox reagents [16,21]. Moreover, the resting enzyme is different for different preparations, depending on the isolation method [16,22,23]. This can also be concluded from the variable position of the Soret absorbance maximum: in some preparations the maximum is positioned at 417 nm, whereas in our preparation (which is a modified Fowler type) it lies at 424 nm [17].

When the enzyme is reduced with excess dithionite and reoxidised with O_2, the Soret band has its absorbance maximum at 426–428 nm. This enzyme form, which is defined primarily by its absorbance spectrum, is called the 'oxygenated' form [13,17,19,24,25].

Antonini et al. [26] found that shortly after reoxidation the enzyme has a higher catalytic activity than the 'resting' enzyme. They defined this more active form as the 'pulsed' enzyme [7,26]. Other enzyme forms, for instance the 'open' and 'closed' conformations according to Refs. 27–29, have also been postulated.

For a long time it was assumed that the 'pulsed' and the 'oxygenated' compounds are identical. Recently, Kumar et al. showed in two publications that this is not the case [11,30]: the enzyme can have its absorbance maximum at 420 nm and yet show the enhanced activity of the 'pulsed' form. They suggested that the 'oxygenated' form is in fact the peroxide complex. Shortly before the appearance of these results, Bickar et al. [9] studied the reaction of H_2O_2 with the 'resting' and 'pulsed' enzyme forms. We reinvestigated the reactions of H_2O_2 with different forms of oxidised cytochrome *c* oxidase, taking into account the results of Kumar et al. [11,30], of which the terminology will be used in this paper.

Materials and Methods

Bovine cytochrome *c* oxidase was isolated according to Refs. 29 and 31; cytochrome *c* was isolated according to Ref. 32.

Cytochrome *c* oxidase was dissolved in 100 mM potassium phosphate (pH 7.4) and 1% Tween-80 to a final concentration that varied from 5 μM for measurements in the Soret region to about 20 μM for measurements at 830 nm. In the rapid-scan/stopped-flow measurements with reduced cytochrome *c* oxidase the buffer also contained 50 mM glucose. The oxidase concentration was determined spectrophotometrically using an absorbance coefficient (red-ox) of 24.0 $mM^{-1} \cdot cm^{-1}$ at 605 nm [33]. All experiments were performed at 20°C.

H_2O_2 (Suprapur) and glucose were from Merck, ascorbic acid (Analar) and sodium dithionite were from British Drug Houses, helium was from Hoekloos, glucose oxidase (from *Aspergillus niger*, Grade I) was from Boehringer Mannheim GbmH.

The titration experiments were performed in a modified Cary-14 spectrophotometer [15] using Thunberg cuvettes. In these experiments anaerobiosis was accomplished by repeated cycles of evacuation and flushing with helium. After anaerobiosis, the enzyme was reduced by addition of excess sodium ascorbate (5 mM) and a small amount of cytochrome *c* (60 nM).

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. For the experiments with reduced cytochrome *c* oxidase reduction was achieved by addition of excess sodium dithionite. Upon transfer of the cytochrome *c* oxidase-solution to one of the stopped-flow reservoirs, the added dithionite will consume the oxygen present in the buffer. The concentration of the dithionite added was not exactly determined for each measurement but was approx. 800 μM. After consumption of the oxygen in the buffer and mixing about 150 μM was left. At this dithionite concentration rereduction will be very slow compared to the reactions studied in this paper. This was checked by monitoring the reaction for some minutes after mixing to make sure no re-reduction took place in that period. The other reservoir of the stopped-flow spectrophotometer contained buffer with 50 mM glucose when oxygen was the electron acceptor. As the experiments were performed at room temperature, the oxygen concentration of the air-equilibrated buffer was taken to be 250 μM [34]. When H_2O_2 was the electron acceptor, the oxygen was converted into H_2O_2 by addition of a small amount of glucose oxidase to the glucose-containing buffer. This methods was previously applied by Bickar et al. [9]. When, in this paper, reference is made to resting cytochrome *c* oxidase, this is the enzyme as isolated. Oxygenated oxidase was prepared by reduction with excess sodium dithionite and reoxidation by vigorously mixing with air immediately before the experiment.

The pulsed enzyme was formed by decay of the oxygenated form. In our stopped-flow experiments we used a different method to prepare the pulsed enzyme which is explained in the Results section. The criterium to discern whether we are dealing with the oxygenated or the pulsed enzyme is the position of the Soret band, which is for our preparation 427 and 424 nm, respectively.

Results

Oxidation of reduced cytochrome c oxidase by hydrogen peroxide

The oxidation of cytochrome *c* oxidase was followed by measuring rapid-scan spectra in the wavelength region from 350 to 900 nm. With O_2 (about 125 μM) as the electron acceptor, the oxidation of cytochrome a_3 was complete and the oxidation of cytochrome *a* almost complete within the mixing time (about 1.5 ms) of the stopped-flow system. This is in accordance with the oxidation rate values reported by other investigators: *k* about 10^5 s^{-1} for oxidation of the cytochrome a_3-Cu_B site and *k* about 10^3 s^{-1} for cytochrome *a* and Cu_A oxidation [2]. The absorbance spectrum of the reoxidised enzyme had a Soret maximum at 424 nm (Fig. 1, dotted line).

When the oxygen was converted into hydrogen peroxide before mixing it with cytochrome *c* oxidase (see Materials and Methods section for details), the reaction was much slower. Fig. 1 shows the absorbance and absorbance difference spectra in the Soret region induced by addition of approximately 125 μM H_2O_2 to reduced cytochrome *c* oxidase. The reaction consisted of only one spectral phase, as can be judged from the fact that the isosbestic point remained nearly constant at about 433 nm throughout the reaction.

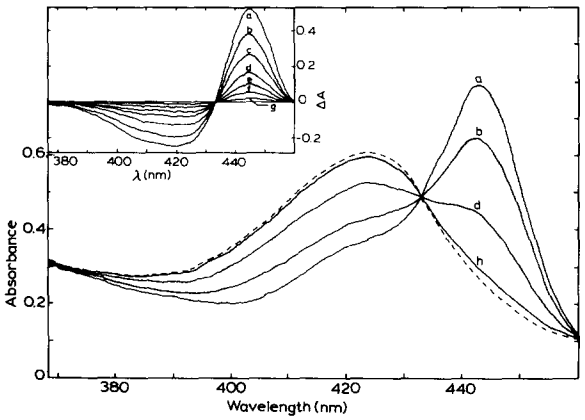


Fig. 1. Kinetic absorbance spectra in the Soret region of the oxidation of reduced cytochrome *c* oxidase by 125 μM H_2O_2. The inset shows the absorbance difference spectra, in which the spectrum obtained after 957 ms was subtracted from all other spectra. Conditions: 5.0 μM cytochrome *c* oxidase, 100 mM potassium phosphate (pH 7.4), 1% Tween-80, 50 mM glucose, sodium dithionite, glucose oxidase. See Materials and Methods for experimental details. (a) 117 ms; (b) 237 ms; (c) 357 ms; (d) 477 ms; (e) 597 ms; (f) 717 ms; (g) 837 ms; (h) 957 ms. The dotted line represents the absorbance spectrum that was obtained 17 ms after the addition of 125 μM O_2 to reduced cytochrome *c* oxidase.

When the peroxide concentration was increased to approx. 5 mM, this was no longer the case: an appreciable part of the absorbance peak at 445 nm had disappeared before the first spectrum was measured (at about 8 ms), while on the other hand a large part of the peak was still present at about 50 ms, when the last spectrum was measured (Fig. 2). Also, the isosbestic point clearly shifted from 429 to 435 nm. These observations indicate that at this peroxide concentration the oxidation of cytochrome a_3 preceded the oxidation of cytochrome a.

This finding is confirmed by rapid-scan spectra in other wavelength regions. Fig. 3 shows the absorbance and absorbance difference spectra in the visible region. The α peak, which is mainly due to cytochrome a [35], decreased with the same slow rate which was observed for the second phase at 445 nm. Fig. 4 shows the absorbance changes upon addition of hydrogen peroxide at 445 nm, 436 nm, 428 nm, 605 nm, 830 nm and 655 nm for several peroxide concentrations. According to Vanneste [35] the absorbance changes at 436 nm are caused by cytochrome a_3 and the absorbance changes at 428 nm are caused by cytochrome a. The absorbance changes at 605 nm are primarily due to cytochrome a; the absorbance changes at 445 nm are caused by both cytochromes (67% cytochrome a_3, 33% cytochrome a). The ab-

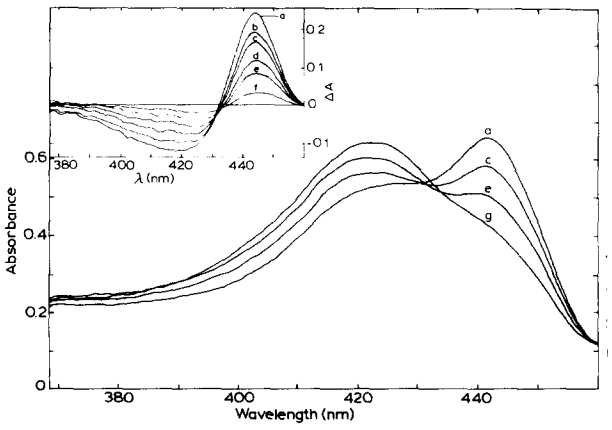


Fig. 2. Kinetic absorbance spectra in the Soret region of the oxidation of reduced cytochrome c oxidase by 5 mM H_2O_2. The inset shows the absorbance difference spectra, in which the spectrum obtained after 50 ms was subtracted from all other spectra. Conditions as in Fig. 1, except that 5 mM H_2O_2 was added and that the cytochrome c oxidase concentration was 4.9 μM. See Materials and Methods for experimental details. (a) 8 ms; (b) 11 ms; (c) 14 ms; (d) 20 ms; (e) 26 ms; (f) 38 ms; (g) 50 ms.

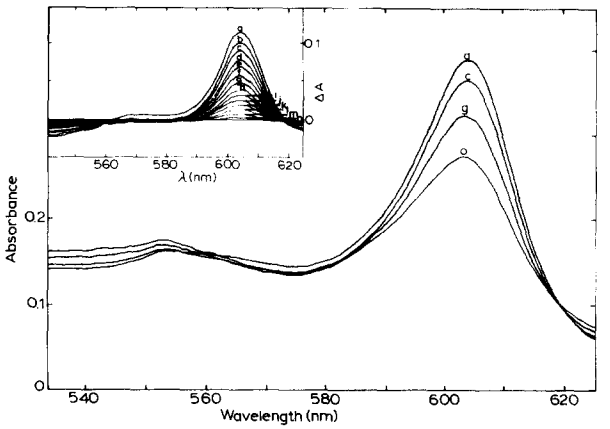


Fig. 3. Kinetic absorbance spectra in the visible region of the oxidation of reduced cytochrome c oxidase by 5 mM H_2O_2. The inset shows the absorbance difference spectra in which the spectrum obtained after 98 ms was subtracted from all other spectra. Conditions as in Fig. 2, except 12.5 μM cytochrome c oxidase. See Materials and Methods for experimental details. (a) 14 ms; (b) 20 ms; (c) 25 ms; (d) 32 ms; (e) 38 ms; (f) 44 ms; (g) 50 ms; (h) 56 ms; (i) 62 ms; (j) 68 ms; (k) 74 ms; (l) 80 ms; (m) 86 ms; (n) 92 ms; (o) 98 ms.

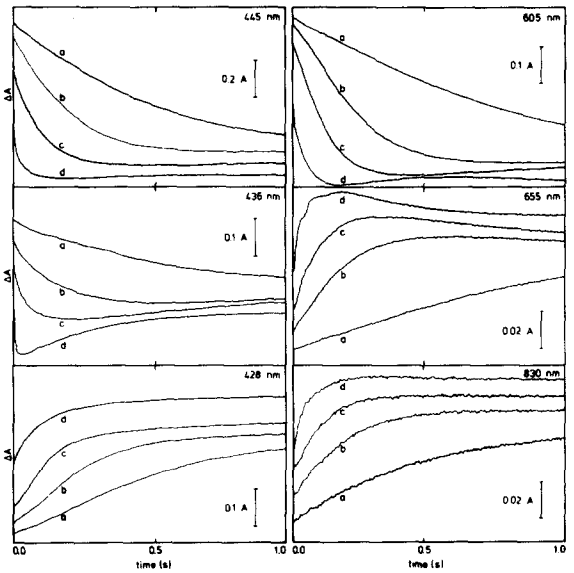


Fig. 4. Absorbance changes at 445 nm, 436 nm, 428 nm, 605 nm, 655 nm and 830 nm induced by the addition of 0.1 mM (a), 0.6 mM (b), 2 mM (c) and 10 mM H_2O_2 (d) to reduced cytochrome c oxidase. Conditions as in Fig. 1. See Materials and Methods for experimental details.

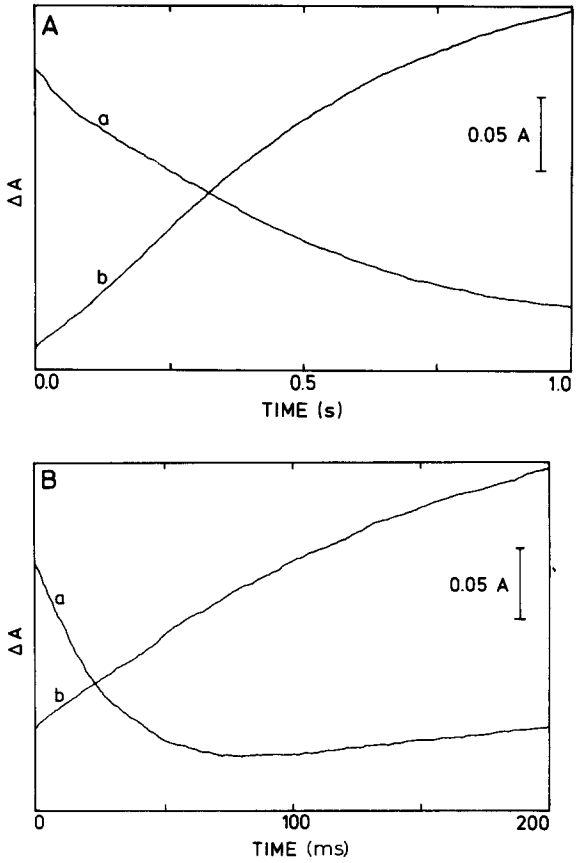


Fig. 5. Absorbance changes at 436 nm (a) and 428 nm (b) induced by addition of H_2O_2 to reduced cytochrome *c* oxidase. Conditions as in Fig. 1. See Materials and Methods for experimental details. (A) 0.1 mM H_2O_2; (B) 2.5 mM H_2O_2.

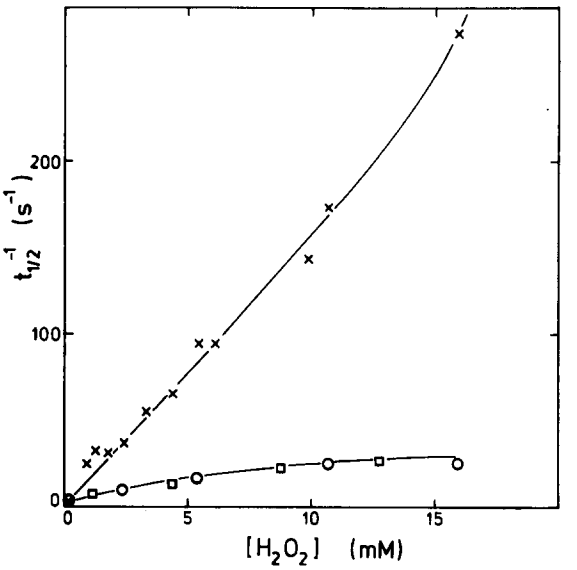


Fig. 6. Dependence of the rate of oxidation of reduced cytochrome *c* oxidase upon the hydrogen peroxide concentrations as observed at 436 nm (×), 605 nm (○) and 830 nm (□). The slow absorbance changes, caused by the formation of the peroxide-complex, were subtracted before the oxidation rates were estimated. Conditions as in Fig. 1. See Materials and Methods for experimental details.

sorbance changes at 830 nm are due to Cu_A [36,37]. The absorbance shoulder at 655 nm in the spectrum of oxidised cytochrome *c* oxidase is believed to be a specific feature of the oxidised cytochrome a_3-Cu_B site when there is interaction between both metal centers [38]. The results show that, while at low peroxide concentrations the oxidation was equally fast at all wavelengths, different rates were measured at higher peroxide concentrations. The oxidation rates at 830 nm, 605 nm, 428 nm and 655 nm were substantially slower than the oxidation rate at 436 nm and the fast phase at 445 nm. This shows that under those conditions cytochrome a_3 is oxidised faster than cytochrome *a* and Cu_A.

The comparatively slow oxidation of cytochrome *a* is illustrated more clearly in Fig. 5, which shows the absorbance changes at 428 nm and 436 nm induced by the addition of 0.13 mM H_2O_2 (Fig. 5A) and 2.5 mM H_2O_2 (Fig. 5B). For 0.13 mM H_2O_2 the oxidation rates of cytochrome *a* and a_3 are similar, while for 2.5 mM H_2O_2 cytochrome a_3 is oxidised faster than cytochrome *a*.

Fig. 6 shows the dependence of rates of oxidation of cytochrome a_3, cytochrome *a* and Cu_A on the peroxide concentration. The oxidation of cytochrome a_3 accelerated with increasing peroxide concentration. The oxidation rate of cytochrome *a* and Cu_A, however, increased until a maximal value of about 20 s^{-1} was reached.

After oxidation of cytochrome *c* oxidase, the enzyme will form a stable complex with H_2O_2 [9–12]. This is a much slower reaction than the oxidation by H_2O_2 [15]. When the time range was extended this slower process could be seen very clearly in the rapid-scan experiments (not shown, but see Fig. 11 and Fig. 12). It also showed up in the stopped-flow traces (Fig. 4). At some wave-

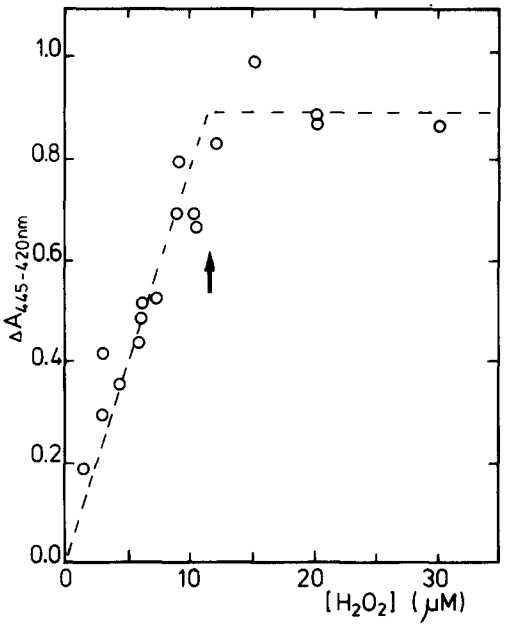


Fig. 7. Titration of reduced cytochrome *c* oxidase with hydrogen peroxide. Plotted is the peak-to-peak amplitude of the absorbance difference spectrum in the Soret region induced by addition of H_2O_2 as a function of the peroxide concentration. Absorbance spectra were measured before and 30 s after addition of H_2O_2. Conditions: 5.7 μM cytochrome *c* oxidase, 100 mM potassium phosphate (pH 7.4), 1% Tween-80, 5 mM sodium ascorbate, 10 nM cytochrome *c*. See Materials and Methods for experimental details. The arrow corresponds to the concentration of hydrogen peroxide at which the absorbance changes reach their maximal value.

lengths this reaction interfered appreciably with the exact determination of the oxidation rate; this was especially so at 436 nm, at which wavelength the oxidation of cytochrome a_3 causes relatively small absorbance changes [35], while H_2O_2-complex formation has its maximum in the absorbance difference spectrum at that wavelength [9]. The result is that at 436 nm the absorbance decrease, due to cytochrome a_3 oxidation, was followed by a much slower absorbance increase, due to $a_3{}^{3+} \cdot H_2O_2$-complex formation.

In order to investigate how many electrons are taken up by H_2O_2 in the oxidation of cytochrome *c* oxidase, the peak-to-peak amplitude of the absorbance difference spectrum in the Soret region of the fully reduced enzyme was measured as a function of the added amount of hydrogen peroxide. The shape of the absorbance difference spectrum was independent of the added amount of hydrogen peroxide for substoichiometric concentrations. For higher peroxide concentrations an increasing contribution of the formation of the peroxide complex could be observed. The result is shown in Fig. 7. The arrow indicates the peroxide concentration at which the absorbance change reached its maximal value if H_2O_2 acts as a two-electron acceptor. The experimental data are consistent with such a behaviour.

Formation of the $a_3{}^{3+} \cdot H_2O_2$ complex

The formation of the $a_3{}^{3+} \cdot H_2O_2$ complex was studied in stopped-flow and rapid-scan experiments after mixing H_2O_2 with fully oxidized resting cytochrome *c* oxidase. At peroxide concentrations higher than 1 mM, complex formation clearly was not a single process. The Soret absorbance peak first rapidly shifted from 423 to 424 nm, and subsequently shifted slowly further to 428 nm (Fig. 8). The spectral contributions of these two processes were somewhat different (Fig. 8 inset). The first phase consisted of an absorbance decrease around 410 nm and an absorbance increase around 432 nm with an isosbestic point at about 420 nm. It must be stressed, however, that

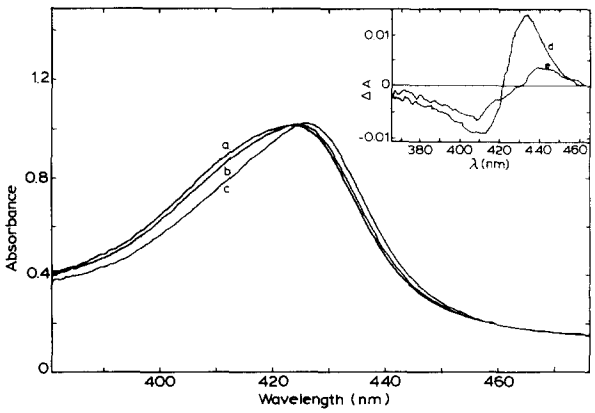


Fig. 8. Kinetic absorbance spectra in the Soret region measured after mixing resting oxidised cytochrome *c* oxidase with 5 mM H_2O_2, (a) 2 s; (b) 17 s; (c) 300 s. The inset shows the absorbance difference spectra that were obtained by subtracting the spectrum at 57 ms from the spectrum at 117 ms (d), and by subtracting the spectrum at 4.4 s from the spectrum at 17.6 s (e). Conditions: 6.0 μM cytochrome *c* oxidase, 100 mM potassium phosphate (pH 7.4), 1% Tween-80. See Materials and Methods for experimental details.

these spectral features were rather variable. The second phase consisted of an absorbance decrease around 408 nm and a small absorbance increase at 438 nm with an isosbestic point at 430 nm. These absorbance changes were sometimes followed by a slow absorbance decrease of the Soret peak, especially when high peroxide concentrations were present, indicating that at longer time scales the enzyme started to decompose.

The rate of complex formation was measured in stopped-flow experiments at a measuring wavelength of 415 nm. We observed at least two phases. Fig. 9 shows the absorbance changes at different peroxide concentrations. From this figure it becomes clear that the rate of the first phase was dependent upon the peroxide concentration, whereas that of the second phase was not. When the apparent rate constant of the first phase was plotted against the peroxide concentration, a straight line was found (Fig. 10). From the slope of this line a rate constant for formation of the complex of 700 $M^{-1} \cdot s^{-1}$ could be calculated. The slow phase had a rate of 0.21 s^{-1}. These two phases were followed by slower ones, but the rates of those could not be measured accurately because they coincided with the overall absorbance decrease in the Soret region that was already mentioned. In general, however, complex formation was complete within 5 min.

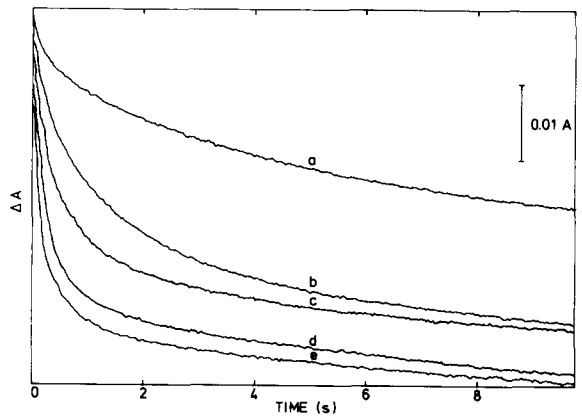


Fig. 9. Absorbance changes at 415 nm induced by addition of H_2O_2 to resting cytochrome *c* oxidase. Conditions as in Fig. 8. See Materials and Methods for experimental details. (a) 0.25 mM H_2O_2; (b) 1 mM H_2O_2; (c) 2.5 mM H_2O_2; (d) 10 mM H_2O_2; (e) 25 mM H_2O_2.

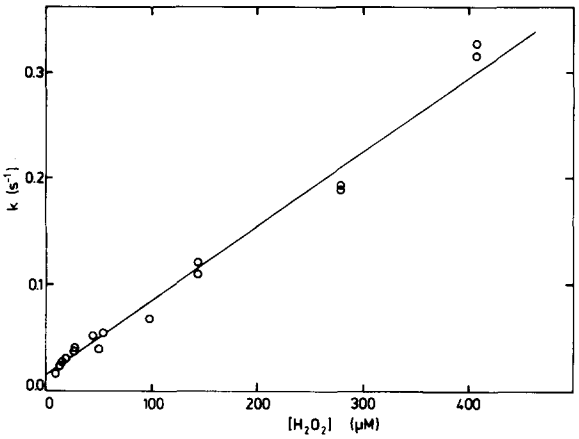


Fig. 10. Dependence of the rate of complex formation of H_2O_2 with oxidised cytochrome *c* oxidase on H_2O_2 concentration. Plotted are the observed rate constants of the first phase detected after mixing H_2O_2 with resting cytochrome *c* oxidase (see text). Conditions as in Fig. 6.

When the enzyme was oxygenated before addition of H_2O_2, the Soret absorbance peak was situated at 427 nm. After addition of H_2O_2, the peak shifted to 428 nm (not shown). After this small red shift no further absorbance changes were detected, apart from the bleaching that was also observed with the resting enzyme. The absorbance difference spectrum was characterised by a decrease at 412 nm, an increase at 434 nm, and an isosbestic point at 424 nm. With the oxygenated enzyme the amplitude of the absorbance changes was extremely variable, an observation that was already made by Bickar et al. [9]. When the rate at 415 nm was measured, it turned out that with the oxygenated enzyme only the first phase was present.

If one allows oxygenated cytochrome *c* oxidase to stand for 20–30 min, the absorbance peak will shift slowly back to 424 nm and the pulsed enzyme is formed [11,30]. When H_2O_2 was added to this compound, the 428 nm absorbance peak was formed, but much faster than with the resting enzyme (not shown). Unfortunately, by forming the pulsed enzyme in this way, one always has a mixture of all three enzyme forms, as the pulsed enzyme (while being formed from the oxygenated compound) will decay to the resting state. In order to evade this problem, dithionite was added to the

reservoir of the stopped-flow apparatus that contained resting cytochrome *c* oxidase. In that case, O_2 will be consumed and the cytochrome *c* oxidase reduced. When the contents of both reservoirs are mixed, O_2 in the H_2O_2-containing reservoir will rapidly re-oxidise the cytochrome *c* oxidase. From the results shown above and from previous reports by other investigators [18] it is known that under these conditions the pulsed enzyme will be formed without prior formation of the oxygenated enzyme. The Soret absorbance spectra obtained in this way are shown in Fig. 11. The Soret peak shifts from 424 to 428 nm; the reaction is characterized by an absorbance decrease at 413 nm, an absorbance increase at 435 nm, and an isosbestic point at 424.5 nm. The reaction, as measured at 415 nm, was completely monophasic and followed first-order kinetics (Fig. 11 inset). Its rate was H_2O_2-dependent and equal to the rate of the first phase that was observed in the reaction of the resting enzyme (700 $M^{-1} \cdot s^{-1}$), but the amplitude of the absorbance change was approx. 4 times as large.

The reaction was also studied in the visible region. In this region the complex formation of the pulsed enzyme form was clearly biphasic. Fig. 12 shows the absorbance spectra measured after

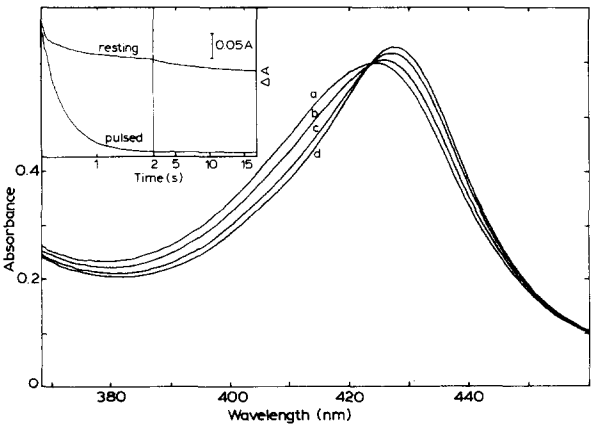


Fig. 11. Kinetic absorbance spectra in the Soret region after mixing pulsed oxidised cytochrome *c* oxidase with 1.5 mM H_2O_2. The inset shows the absorbance changes at 415 nm induced by the addition of 3.7 mM H_2O_2 to both resting and pulsed cytochrome *c* oxidase. Conditions: 4.0 μM cytochrome *c* oxidase, 100 mM potassium phosphate (pH 7.4), 1% Tween-80, sodium dithionite. See text for experimental details. (a) 432 ms; (b) 872 ms; (c) 1752 ms; (d) 3512 ms.

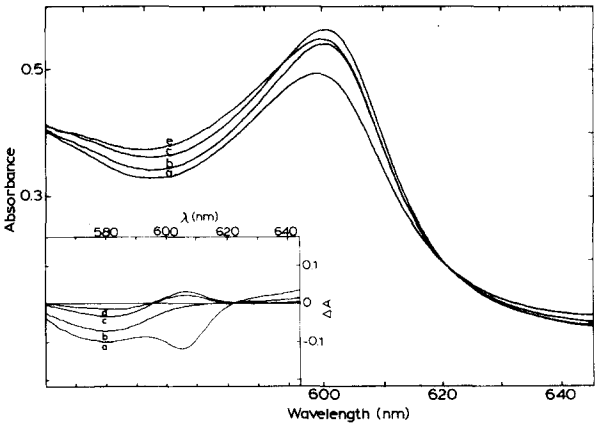


Fig. 12. Kinetic absorbance spectra in the visible region after mixing of pulsed oxidised cytochrome *c* oxidase with 7.5 mM H_2O_2. The inset shows the absorbance difference spectra, in which the spectrum obtained after 1757 ms was subtracted from all other spectra. Conditions as in Fig. 8 except 15 μM cytochrome *c* oxidase See text for experimental details. (a) 217 ms; (b) 437 ms; (c) 877 ms; (d) 1317 ms; (e) 1757 ms.

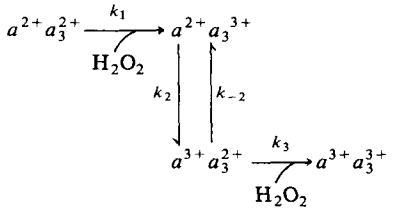
addition of 7.5 mM H_2O_2 to pulsed cytochrome *c* oxidase. The first phase consists of an absorbance increase at 605 nm and a pronounced shoulder at 580 nm, with an isosbestic point at 624 nm. Subsequently, a blue shift was observed resulting in an absorbance increase at 582 nm, a decrease at 607 nm and an isosbestic point at 595 nm. Rapid-scan measurements at 580 nm and at 605 nm confirmed this; at 605 nm the two phases induced absorbance changes of opposite sign, as has already been reported by Bickar et al. [39]. The first phase was peroxide-dependent and occurred simultaneously with the absorbance changes in the Soret region. The second phase was peroxide-independent and had a rate of 0.9 s^{-1}.

With the oxygenated enzyme, similar absorbance changes could be observed, but with a much smaller amplitude; on most occasions the second phase was relatively more pronounced than the first phase in comparison to the absorbance changes observed with the pulsed enzyme. With the resting enzyme, the fast phase was again observed with an amplitude 4 times smaller than with the pulsed enzyme. The second phase with this enzyme conformation had a rate of 0.2 s^{-1}, which is similar to that observed for the second phase in the Soret region. Slower absorbance

changes occurred, but could not be determined accurately.

Discussion

In an earlier paper [15] we reported that reduced cytochrome *c* oxidase is oxidised by hydrogen peroxide and that this reaction takes place at the oxygen-binding site. We showed that the rate of cytochrome a_3-oxidation is linearly dependent on the hydrogen peroxide concentration. These observations are confirmed here. Scheme I shows the reaction sequence of the oxidation of cytochrome *c* oxidase by hydrogen peroxide:



Scheme I

In this model $k_1 = k_3$ as was established by us previously [15], when we showed that the oxidation rates of the fully reduced and of the partially reduced enzyme are equal.

When low concentrations of peroxide were added we found that the absorbance changes occurred with the same rate at all wavelengths (and could be fitted as a first-order reaction), so cytochrome *a* and cytochrome a_3 were oxidised simultaneously. This implies that $k_2, k_{-2} \gg k_1[H_2O_2], k_3[H_2O_2]$. It also implies that $k_2 = k_{-2}$, i.e., that cytochrome *a* and a_3 have about the same redox potential. If cytochrome *a* had a lower redox potential than cytochrome a_3, the result would be that cytochrome *a* was oxidised first and cytochrome a_3 subsequently; if cytochrome *a* had a higher redox potential, cytochrome a_3 would have been oxidised first and cytochrome *a* oxidation would have exhibited both a lag phase and a slower rate. The conclusion that $k_2 = k_{-2}$ is in agreement with the results of previous potentiometric studies [40–42]. When $k_1 = k_3$, $k_2 = k_{-2}$ and $k_2, k_{-2} \gg k_1[H_2O_2], k_3[H_2O_2]$ it can be derived that the reaction will be first order with an observed pseudo-first-order rate constant $k_{obs} = \frac{1}{2}k_1[H_2O_2]$. The observed rate constant we measured at 444, 428 and 436 nm was approx. $1.0 \cdot 10^4\ M^{-1} \cdot s^{-1}$. This yields a value for k_1 of $2 \cdot 10^4\ M^{-1} \cdot s^{-1}$, which is in agreement with the values reported previously [15].

For high peroxide concentrations the oxidation of cytochrome a_3 is faster than cytochrome *a*- and Cu_A-oxidation. This means that then $k_1[H_2O_2]$ becomes greater than k_2. From this we conclude that the intermediate electron transfer is slow.

In Ref. 15 we already reported that the rate of electron transfer from cytochrome *a* to a_3 was low (0.5–5 s^{-1}). That value was a rough estimation and its significance was not clear, as it was measured under conditions where CO was present in the sample. In this paper we confirm that the internal electron transfer is slow and a more accurate estimation of the rate constant can be given: between 15 and 25 s^{-1}. Furthermore, the experimental approach used in this paper enabled us to compare directly the internal transfer rates with O_2 and with H_2O_2 as the electron acceptor. Our results clearly show that with oxygen the transfer of electrons from cytochrome *a* (and Cu_A) to the cytochrome a_3-Cu_B site was much faster (700 s^{-1}) than with H_2O_2 (15–25 s^{-1}). This difference can be explained in several ways. It could be that H_2O_2 in some way inhibits internal electron transfer. However, it is more likely that the fast rate which is found during turnover [43–45] and in oxidative pre-steady-state measurements [46,47] is a specific feature of the oxygen reaction. The most appealing explanation for such a behaviour is that in the reaction with oxygen the internal electron-transfer rate is enhanced by the peroxo-intermediate that is formed when oxygen is bound to the a_3-Cu_B site and has taken up two electrons, as has been proposed previously [7,48]. Such an intermediate is not formed in the reaction with peroxide as studied in this paper.

We have not detected any intermediates in the reaction. At the highest concentrations applied the oxidation rate still increases with the peroxide concentrations. The value for k_{obs} that can be derived for these concentrations (25 mM and higher) are lower than expected (300 s^{-1} instead of the calculated 500 s^{-1}) but we ascribe this to the fact that at these rates a considerable part of

the reaction occurs within the mixing time of the apparatus; this is likely to induce errors in the calculations.

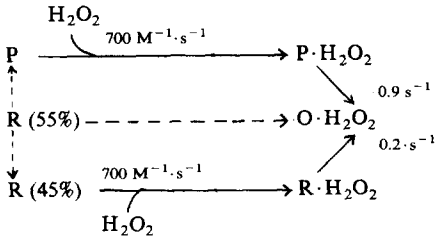
The distribution of the fast and slow phases in the oxidation process over the wavelength range fits the absorption spectra of cytochrome *a* and a_3 according to Vanneste [35]. The 655 nm absorbance shoulder, which is generally believed to be caused by a charge transfer within the cytochrome a_3-Cu_B site [38], is not formed simultaneously with cytochrome a_3 oxidation but at a slower rate. This suggests that the strong interaction between cytochrome a_3 and Cu_B that is characteristic for the oxidised cytochrome a_3-Cu_B site is not established simultaneously with cytochrome a_3 oxidation. Another explanation would be that Cu_B is oxidised more slowly than cytochrome a_3, but this would imply that a one-electron reduced H_2O_2-intermediate accumulates during the reaction. The absorbance spectra between 350 nm and 900 nm show no evidence for such an intermediate.

H_2O_2 reacted readily with fully oxidised cytochrome *c* oxidase in the pulsed conformation; the rate of H_2O_2 binding was 700 $M^{-1} \cdot s^{-1}$. The experiments performed in the visible region show that H_2O_2 binding was followed by at least one conformational change with a rate of 0.9 s^{-1}.

The results obtained with the resting enzyme are more complicated. One might argue that the pulsed enzyme is the only enzyme form reacting with H_2O_2, if one assumes that the fast phase is caused by a small fraction of pulsed cytochrome *c* oxidase present in the resting state. The slower phases would then represent the transition of the resting to the pulsed conformation. However, this would imply a resting-to-pulsed transition rate of 0.2 s^{-1} and thus an even higher value for the pulsed-to-resting transition. This is much faster than the actual decay time of the pulsed enzyme form, about 30 min [30], can account for. If our observation that complex formation is complete within 5 min is correct, this would also be true for the slower phases. Also the differences between the spectral features of the fast phase of the resting enzyme reaction and of the pulsed enzyme reaction, observed in the Soret region, indicate that they represent different reactions. Finally, the spectral features of the slow phase observed with the resting enzyme (decrease at 408 nm, a small absorbance increase at 438 nm with an isosbestic point at 430 nm) show a striking resemblance to the absorbance differences that Kumar et al. [11] ascribe to the resting-to-pulsed transition. This strongly indicates that complex formation has occurred before this slow phase. From these observations we conclude that at least 45% of the resting enzyme reacted with H_2O_2 with the same rate as the pulsed enzyme, inducing similar but not identical absorbance changes. The transition to the pulsed conformation was accelerated by the formation of the peroxide complex to a rate of 0.21 s^{-1}. The first and second phases together only accounted for approx. 45% of the absorbance change observed with the pulsed enzyme. The remainder of the enzyme reacted more slowly. The results confirm that resting cytochrome *c* oxidase is a heterogeneous mixture of several enzyme forms.

Our results with the oxygenated enzyme show that no other reactions could be detected than those found with the pulsed enzyme, but with much smaller amplitudes. We share the view of Kumar et al. [11,30] and other investigators that the enzyme after oxygenation is, in fact, a peroxide complex. The observed reactions can be explained by partial conversion of the oxygenated enzyme to the pulsed form in the time interval between oxygenation and addition of H_2O_2 to the enzyme. This would also account for the large variability of the absorbance changes with this enzyme form observed by us and by Bickar et al. [9]. The fact that in the visible region the slow phase was more prominent than the fast phase as compared to the pulsed enzyme suggests that in its decay to the pulsed conformation the same intermediate was formed that is seen in H_2O_2 complex formation. Most of our results are summarised in Scheme II.

A comparison of our results with those reported by Bickar et al. [39] shows many similarities but also some differences. They report that both with 'pulsed' and resting cytochrome *c* oxidase the reaction occurs in three H_2O_2-dependent phases, the only differences between both compounds lying in the amplitude of the absorbance changes which is three times greater with the 'pulsed' enzyme. We do not observe their first



Scheme II. Model for the reaction of pulsed and resting cytochrome *c* oxidase with H_2O_2. P, pulsed cytochrome *c* oxidase; R, resting cytochrome *c* oxidase; O, oxygenated cytochrome *c* oxidase.

phase; this phase, which is not much faster than their second phase (our first phase) and only accounts for 15% of the sum of both phases, may be missing in our preparation. Also they report this phase to be peroxide-dependent, whereas in our experiments it is not. Moreover, they do not discriminate between the slow phases obtained with the resting and pulsed conformation of cytochrome *c* oxidase, whereas our results indicate that they belong to quite different processes occurring at different rates, as is evident from the measurements in the Soret region of the optical spectrum where no slow phase is detected with pulsed oxidase.

Acknowledgements

The authors thank Dr. B.F. van Gelder for his interest and reading the manuscript. The study was supported in part by grants from the Netherlands Organization for the Advancement of Pure Research (Z.W.O.) under auspices of the Netherlands Foundation for Chemical Research (S.O.N.).

References

1 Van Gelder, B.F. and Muijsers, A.O. (1966) Biochim. Biophys. Acta 118, 47–57
2 Greenwood, C. and Gibson, Q.H. (1967) J. Biol. Chem. 242, 1782–1787
3 Chance, B., Leigh, J.S., Jr. and Waring, A. (1977) in Structure and Function of Energy-Transducing Membranes (Van Dam, K. and Van Gelder, B.F., eds.), BBA Library 14, pp. 1–10, Elsevier, Amsterdam
4 George, P. (1965) in Oxidases and Related Redox Systems (King, T.E., Mason, H.S. and Morrison, M., eds.), pp. 3–36, Wiley, New York
5 Malmström, B.G. (1974) Q. Rev. Biophys. 6, 389–431
6 Chance, B., Saronio, C. and Leigh, J.S., Jr. (1975) J. Biol. Chem. 250, 9226–9237
7 Brunori, M., Colosimo, A., Rainoni, G., Wilson, M.T. and Antonini, E. (1979) J. Biol. Chem. 254, 10769–10775
8 Hill, B.C. and Greenwood, C. (1983) Biochem. J. 215, 659–667
9 Bickar, D., Bonaventura, J. and Bonaventura, C. (1982) Biochemistry 21, 2661–2666
10 Orii, Y. (1982) J. Biol. Chem. 257, 9246–9248
11 Kumar, C., Naqui, A. and Chance, B. (1984) J. Biol. Chem. 259, 2073–2076
12 Wrigglesworth, J.M. (1984) Biochem. J. 217, 715–719
13 Orii, Y. and Okunuki, K. (1963) J. Biochem. 54, 207–213
14 Brunori, M., Bickar, D., Bonaventura, J. and Bonaventura, C. (1985) J. Biol. Chem. 260, 7165–7167
15 Gorren, A.C.F., Dekker, H. and Wever, R. (1985) Biochim. Biophys. Acta 809, 90–96
16 Brudvig, G.W., Stevens, T.H., Morse, R.H. and Chan, S.I. (1981) Biochemistry 20, 3912–3921
17 Muijsers, A.O., Tiesjema, R.H. and Van Gelder, B.F. (1971) Biochim. Biophys. Acta 234, 481–492
18 Rosèn, S., Brändèn, R., Vänngård, T. and Malmström, B.G. (1977) FEBS Lett. 74, 25–30
19 Shaw, R.W., Hansen, R.E. and Beinert, H. (1978) J. Biol. Chem. 253, 6637–6640
20 Petersen, L.C. and Cox, R.P. (1980) Biochim. Biophys. Acta 590, 128–137
21 Naqui, A., Kumar, C., Ching, Y., Powers, L. and Chance, B. (1984) Biochemistry 23, 6222–6227
22 Lemberg, M.R. (1969) Physiol. Rev. 49, 48–121
23 Kornblatt, J.A. and Hui Bon Hoa, G. (1982) Biochemistry 21, 5439–5444
24 Okunuki, K., Hagihari, B., Sekuzu, I. and Horio, T. (1958) Proc. Int. Symp. Enzyme Chem. Tokyo, Kyoto (Ichihara et al., eds.), pp. 264–272, Maruzen, Tokyo
25 Orii, Y., and Okunuki, K. (1963) J. Biochem. 53, 489–499
26 Antonini, E., Brunori, M., Colosimo, A., Greenwood, C.and Wilson, M.T. (1977) Proc. Natl. Acad. Sci. USA 74, 3128–3132
27 Van Buuren, K.J.H., Zuurendonk, P.F., van Gelder, B.F. and Muijsers, A.O. (1972) Biochim. Biophys. Acta 256, 243–257
28 Jones, M.G., Bickar, D., Wilson, M.T., Brunori, M., Colosimo, A. and Sarti, P. (1984) Biochem. J. 220, 57–66
29 Jensen, P., Wilson, M.T., Aasa, R. and Malmstrom, B.G. (1984) Biochem. J. 224, 829–837
30 Kumar, C., Naqui, A. and Chance, B. (1984) J. Biol. Chem. 259, 11668–11671
31 Hartzell, C.R., Beinert, H., Van Gelder, B.F. and King, T.E. (1978) Methods Enzymol. 53, 54–66
32 Margoliash, E. and Walasek, O.F. (1967) Methods Enzymol. 10, 339–348
33 Van Gelder, B.F. (1966) Biochim. Biophys. Acta 118, 36–46
34 Loomis, A.G. (1928) in International Critical Tables, Vol. III (Washburn, E.W., ed.), p. 257, McGraw-Hill, New York

35 Vanneste, W.H. (1966) Biochemistry 5, 838–848
36 Beinert, H., Shaw, R.W., Hansen, R.E. and Hartzell, C.R. (1980) Biochim. Biophys. Acta 591, 458–470
37 Boelens, R. and Wever, R. (1980) FEBS Lett. 116, 223–226
38 Beinert, H., Hansen, R.E. and Hartzell, C.R. (1976) Biochim. Biophys. Acta 423, 339–355
39 Bickar, D., Lehninger, A., Brunori, M., Bonaventura, J. and Bonaventura, C. (1985) J. Inorg. Biochem. 23, 365–372
40 Nicholls, P. and Petersen, L.C. (1974) Biochim. Biophys. Acta 357, 462–467
41 Wikström, M.K.F., Harmon, H.J., Ingledew, W.J. and Chance, B. (1976) FEBS Lett. 65, 259–277
42 Carithers, R.P. and Palmer, G. (1981) J. Biol. Chem. 256, 7967–7976
43 Lemberg, R. and Barrett, J. (1973) in Cytochromes, pp. 17–57, Academic Press, New York
44 Nicholls, P. and Chance, B. (1974) in Molecular Mechanisms of Oxygen Activation (Hayashi, O., ed.), pp. 479–534, Academic Press, New York
45 Wilms, J., van Rijn, J.L.M.L. and Van Gelder, B.F. (1980) Biochim. Biophys. Acta 593, 17–23
46 Gibson, Q.H., Greenwood, C., Wharton, D.C. and Palmer, G. (1965) J. Biol. Chem. 240, 888–894
47 Gibson, Q.H. and Greenwood, C. (1965) J. Biol. Chem. 240, 2694–2698
48 Orii, Y. (1984) J. Biol. Chem. 259, 7187–7190