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BIOCHEMICAL AND BIOPHYSICAL STUDIES ON CYTOCHROME aa_3

IV. SOME PROPERTIES OF OXYGENATED CYTOCHROME aa_3

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SUMMARY

1. Our data support the idea that the so-called 'oxygenated' cytochrome aa_3 is no oxygenated or peroxidic compound, but a different conformational state of the oxidized enzyme.

2. The conversion of oxygenated to oxidized cytochrome *c* oxidase obeys first-order kinetics ($k = 5 \cdot 10^{-4}$ sec^{-1}) and has an activation energy of 11.5 kcal/mole.

3. Under conditions where the oxidized enzyme is rapidly reduced by NADH *plus* phenazine methosulphate, the oxygenated form does not react at all.

4. By means of an anaerobic titration technique the oxygenated form, like the oxidized enzyme, was shown to consume 4 electron equivalents per molecule (or 2 per haem) for complete reduction.

5. The addition under anaerobic conditions of only one electron per mole oxidized cytochrome aa_3, followed by aeration, results in nearly quantitative formation of the oxygenated compound.

6. Aeration experiments with partly and fully reduced cytochrome aa_3 indicate that only the latter is immediately oxidized by oxygen. In both cases the final product is the oxygenated compound.

INTRODUCTION

The lipoprotein cytochrome *c* oxidase (EC 1.9.3.1) catalyses the oxidation of ferrocytochrome *c* by molecular oxygen. The functional unit, containing two haem groups (called *a* and a_3) and two copper atoms[1,2], can take up 4 electrons on reduction.

'Oxygenated' cytochrome *c* oxidase, discovered by OKUNUKI *et al.*[3], is spectroscopically different from the ferric cytochrome aa_3 as shown by several investigators[4-7]. A detailed comparison has been presented in a previous paper of this series[8]. The word 'oxygenated' originates from the concept of an enzyme–oxygen complex[4] but evidence has since accumulated that the compound is of a different nature[9]. The name, however, has been retained by workers in the field and is also used in this paper.

The final proof for the existence of an oxygenated compound, as stated by

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WITTENBERG and co-workers[10] in their study on oxyperoxidase, demands that at least one of the following criteria is met: (a) proof that the compound retains the oxygen molecule consumed in its formation as well as the 4 oxidizing equivalents or (b) proof that the compound dissociates with production of oxygen. No case is known where these conditions are fulfilled by an enzyme, although there is in some cases considerable evidence in favour of the existence of an oxygenated compound[10–12].

The existence of a truly oxygenated cytochrome aa_3 is unlikely[13,14], at least if we restrict our definition to iron-coordinated oxygen, thereby excluding the oxygen-stabilized conformation of oxidized cytochrome aa_3 described in a previous paper[8].

The oxygenated form of cytochrome aa_3 reverts spontaneously to the ferric enzyme[15]; the kinetic aspects of this reaction are reported in this paper, as well as the determination of the number of reducing equivalents taken up by oxygenated cytochrome aa_3 upon reduction. Some of the results of these studies have been published in a preliminary form[16].

EXPERIMENTAL

Enzyme

Cytochrome *c* oxidase was isolated from beef heart muscle preparation and purified by the method of FOWLER *et al.*[17] as extended by MCLENNAN AND TZAGOLOFF[18]. The preparations contained 4–5 μmoles cytochrome aa_3 per gram protein (or 8-10 μmoles haem *a* per gram protein), with, as parameter for reducibility, γ-band (reduced) : γ-band (oxidized), 1.30–1.37. The positions of the γ-bands of the oxidized and oxygenated enzyme were at 423–424 nm and 428 nm, respectively. The YONETANI[19]-type preparation used in some experiments contained 3.6 μmoles aa_3 per gram protein; the γ-band was at 423 nm and the ratio γ-band (reduced) : γ-band (oxidized) was 1.24.

The enzyme concentration was calculated with the millimolar absorbance coefficient for reduced *minus* oxidized cytochrome aa_3 at 605 nm of 24.0 $mM^{-1} \cdot cm^{-1}$ (ref. 20).

Spectra and kinetic measurements were made with Cary 14, Cary 15 and Zeiss M4Q spectrophotometers.

Oxygenation procedure

Unless otherwise stated the standard method included evacuation of the cooled sample and reduction for 20–30 min by a 10-fold excess of $Na_2S_2O_4$. Air was then passed through the solution for 15 min. In some experiments, the oxygenated compound was formed by passing $Na_2S_2O_4$-reduced enzyme through a Sephadex G 25 column[13] or by addition of H_2O_2 (ref. 21).

Anaerobic titration with NADH

Optical Thunberg cells were used, fitted with a hollow stopper with separate compartments for NADH and phenazine methosulphate. During a period of 8 min the tube was alternatively evacuated and filled with argon about 30 times. The cell was cooled in ice and removal of air was facilitated by sharp knocking. The argon used for flushing was freed from traces of oxygen by passing through a column of alkaline pyrogallol. To accelerate the reaction of oxygen with pyrogallol traces of iron and

copper salts were added. Correction for the water evaporated in the Thunberg cuvettes was made by weighing the complete cell before and after the evacuation.

Chemicals

Tween-80, phenazine methosulphate and NADH were purchased from Sigma, the other chemicals were from British Drug Houses, Analar grade. The concentration of NADH was determined with the millimolar absorbance coefficient of 6.22 $mM^{-1} \cdot cm^{-1}$ at 340 nm.

RESULTS

Oxygenation with hydrogen peroxide

In agreement with earlier observations[22,9,21], it was found that oxygenation results from the action of H_2O_2 on oxidized cytochrome aa_3. For complete conversion, however, high concentrations of peroxide are necessary. Ten moles H_2O_2 per mole aa_3 cause only 60 % oxygenation, as seen from the γ-band red shift and the absorbance increase at 432 and 605 nm. A further increase in the H_2O_2 concentration leads to an irreversible change in the enzyme.

Decomposition of oxygenated cytochrome aa_3

All authors agree on the spontaneous conversion of the oxygenated into oxidized preparations but the kinetic aspects of this process have not been investigated. The reaction can easily be followed at 432 nm (near the isosbestic point of reduced and oxidized aa_3) or at 605 nm. The millimolar absorbance coefficients (oxygenated *minus* oxidized) are 26 and 3.0 $mM^{-1} \cdot cm^{-1}$ at 432 and 605 nm, respectively (Fig. 1 of ref. 8).

TABLE I

FIRST-ORDER RATE CONSTANTS FOR THE CONVERSION OF OXYGENATED TO OXIDIZED CYTOCHROME aa_3

Preparation[a]	*Oxygenation method*[b]	λ(*nm*)[e]	*Temp.* (°C)	$10^4 \times k$ (sec^{-1})
F 35	H_2O_2	432	23	4.7
M 115	H_2O_2	432	24	5.4
F 38	$Na_2S_2O_4$/air	605	22	4.5
F 44	$Na_2S_2O_4$/air	605	25	6.1
F 56	$Na_2S_2O_4$/air	605	25	5.8
F 56	$Na_2S_2O_4$/air	605	20	3.8
F 56	$Na_2S_2O_4$/column[c]	605	20	8.7
F 56	$Na_2S_2O_4$/column[d]	605	20	3.8

[a] The capitals F and M indicate Fowler and Yonetani type of enzyme preparations, respectively.

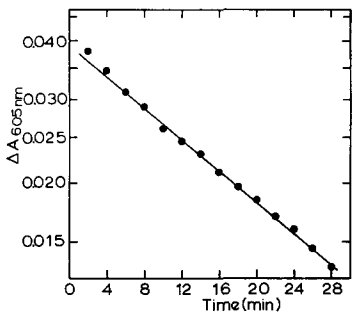
[b] The H_2O_2 and $Na_2S_2O_4$/air procedures are described under EXPERIMENTAL and in the legends of Figs. 1 and 2.

[c] Oxygenation by passing the reduced sample through a short Sephadex G 25 column, equilibrated at 8 °C with 50 mM potassium phosphate (pH 7.2) and 26 mM cholate.

[d] Dithionite oxidation products (prepared by prolonged aeration of a $Na_2S_2O_4$ solution until the 350–400 nm absorbance had disappeared) in a final concentration of 1 mM were added to the oxygenated enzyme sample immediately after leaving the G 25 column.

[e] The wavelengths chosen for measuring the absorbance change associated with the decomposition of oxygenated cytochrome aa_3 were not exactly the peaks in the spectrum oxygenated *minus* oxidized.

From the constant isosbestic points in spectra taken at intervals during the decomposition it may be concluded that no spectroscopically visible intermediates occur during this conversion.



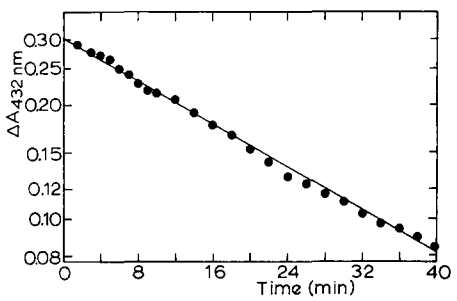


Fig. 1. Decomposition of oxygenated cytochrome aa_3 measured by decline of $A_{605\ nm}$. The difference between $A_{605\ nm}$ at time t and the final value is plotted on the ordinate. Zero time is fixed at the end of the 15-min aeration of the $Na_2S_2O_4$-reduced sample. 33 μM cytochrome aa_3, 100 mM potassium phosphate (pH 7.2), 0.7% cholate, light path 1 cm, temperature 25 °C.

Fig. 2. Decomposition of oxygenated cytochrome aa_3 measured by decline of $A_{432\ nm}$. The ordinate gives the difference between $A_{432\ nm}$ at time t and the final value. Oxygenation was performed by addition of 59 μM H_2O_2 to 20 μM oxidized cytochrome aa_3 (at t, zero) in 37 mM Tris sulphate (pH 8.0), 0.4% Tween 80. Light path 2 mm, temperature 24 °C.

The decomposition reaction of the oxygenated compound as followed at 605 or 432 nm obeys first-order kinetics (Figs. 1 and 2) fairly closely (but see later). Since the rate constants at both wavelengths are the same (Table I), the small 605 nm changes are not due to a slow oxidation of traces of reduced cytochrome aa_3. From observations at 578 nm, a peak in the difference spectrum oxygenated *minus* oxidized aa_3, also the same rate constants were calculated (not shown in Table I). The data summarized in Table I show that the first-order rate constants for Fowler and Yonetani type preparations are the same. Furthermore the k value is not dependent on the choice of $Na_2S_2O_4$/air or H_2O_2 for oxygenation. However, the decomposition rate of the oxygenated compound produced by the $Na_2S_2O_4$/Sephadex method is more than twice as high as that of the $Na_2S_2O_4$/air product. It is concluded that dithionite oxidation products inhibit the conversion of oxygenated cytochrome aa_3 prepared by this method into oxidized cytochrome aa_3. This is supported by the decrease in decomposition velocity observed when dithionite oxidation products are added to an oxygenated sample prepared by the column method (last lines of Table I). In agreement with LEMBERG AND STANBURY[9] it was found that in most experiments the conversion rate is insensitive to catalase. This suggests the traces of H_2O_2 present in the dithionite oxidation products are not the inhibiting agent. The product of the reaction of oxidized cytochrome aa_3 with excess H_2O_2 has the same stability as the product obtained with dithionite and air. No explanation can be offered for this apparent coincidence.

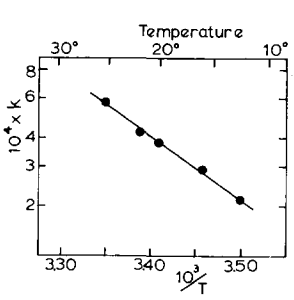
The straight-line Arrhenius plot of log k *versus* $1/T$ (Fig. 3) between 12 and 25 °C corresponds to a Q_{10} for the reaction of 2.15 and an activation energy of 11.5 kcal·mole^{-1}.

Oxygenation after reduction by NADH

When NADH is used as reducing agent in the presence of a catalytic amount of phenazine methosulphate under anaerobic conditions, subsequent aeration also produces the oxygenated compound. The first-order rate constant for the decomposition to the oxidized form is $8 \cdot 10^{-4}$–$15 \cdot 10^{-4}$ sec^{-1} at 20°C, slightly higher than is observed with dithionite. The anaerobic reduction procedure permits the use of stoicheiometric amounts of reducing agent or even less. Because there is no excess reducing agent to be oxidized the aeration time can be shortened from the usual 15 min to 10–20 sec.

The course of events taking place on aeration as observed at 605 nm may tentatively be summarized as a sequence of three reactions. First there is a very rapid reoxidation of the enzyme to the oxygenated form within the aeration time. Not only the absolute but also the relative absorbance decrease caused by this reaction is strongly dependent on the number of reducing equivalents added per mole of enzyme. There is almost no rapid change when only one electron had been added. When the enzyme had been fully reduced by 4 electrons the contribution of the first reaction to the total absorbance difference at 605 nm is 80 %.

The initial rapid formation of the oxygenated form is followed by the slower Reactions 2 and 3. The experimental results as shown in the upper curve of Fig. 4, where the enzyme had been reduced by 1 electron equivalent can be approximated by two simultaneous first-order reactions. The rate constants are about 10^{-2} sec^{-1} for Reaction 2 and 10^{-3} sec^{-1} for Reaction 3. Reaction 3 is identified as the normal conversion reaction from oxygenated to oxidized cytochrome aa_3, since the reaction accounts for 10 % of the total absorbance difference at 605 nm between the fully



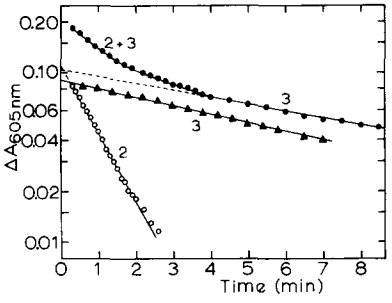


Fig. 3. Arrhenius plot of the dependence of the first-order rate constant for the conversion of oxygenated to oxidized cytochrome aa_3 on the temperature. Oxygenation by the $Na_2S_2O_4$/air method of 30–36 μM cytochrome aa_3 in 43 mM potassium phosphate (pH 7.2), 1 % cholate. The abscissa gives the reciprocal absolute temperature, at the upper side the corresponding temperature (in °C) is plotted. The value at each temperature is the mean of 5 experiments.

Fig. 4. Time course of Reactions 2 and 3 after aeration of cytochrome aa_3, partially or fully reduced with NADH under anaerobic conditions. The ordinate gives the difference between $A_{605\ nm}$ at time t and the final value. Zero time marks the beginning of the 15-sec aeration of the Thunberg cuvette. The fast Reaction 1 is not seen on this time scale. 232 μM cytochrome aa_3 in 75 mM Tris sulphate (pH 7.2). Cholate is present but the concentration was not determined, 4.8 μM phenazine methosulphate, light path 2 mm, temperature 22°C. ▲—▲, aeration after reduction by 448 μM NADH (or 3.9 e^-/aa_3). Only Reaction 3 is seen ($k = 1.9 \cdot 10^{-3}$ sec^{-1}). ●—●, aeration after reduction by 112 μM NADH (or 1.0 e^-/aa_3). Both Reaction 2 ($k = 1.5 \cdot 10^{-2}$ sec^{-1}) and Reaction 3 ($k = 1.6 \cdot 10^{-3}$ sec^{-1}) are visible. Reaction 2 (○—○) was approximated by plotting the absorbance difference between the measured tracing (●—●) and the extrapolated values for the Reaction 3 alone (dashed line). The absorbance difference caused by Reaction 2 as well as by Reaction 3 is 9–10 % of the $\Delta A_{605\ nm}$ (fully reduced *minus* oxidized) in this case.

reduced and oxidized enzyme and shows the known rate constant of 10^{-3} sec^{-1}. Furthermore the γ-peak of the enzyme at the end of Reaction 2 is at 426–427 nm. It may be seen that the anaerobic addition of only one electron per mole oxidized aa_3 (*i.e.* 25 % reduction) is already sufficient to attain the maximal conversion to the oxygenated form upon aeration. Under our conditions this corresponds to about 80 % oxygenation, as calculated with the millimolar absorbance coefficient of 3.0 $mM^{-1} \cdot cm^{-1}$ at 605 nm (Fig. 1 of ref. 8).

The faster Reaction 2 is of a quite different nature and has a half-time of about 1 min. The total absorbance change associated with Reaction 2 as calculated by zero-time extrapolation from plots of the type shown in Fig. 4, but not the rate constant, is strongly dependent on the previous redox state of the enzyme. A maximum of about 15 % of the ΔA value from the difference spectrum fully reduced *minus* oxidized is reached when half-reduced aa_3 (2 electrons added per mole aa_3) is aerated (not shown). Reaction 2 is barely visible on aeration of fully reduced aa_3 (4 electrons) as can be seen in Fig. 4 (triangles). Reaction 2 is under further investigation as it may represent a slow oxidation reaction of cytochrome *a*. This seems to confirm the hypothesis of ANTONINI and coworkers[23] that only the fully reduced enzyme is able to react rapidly with oxygen. Both first-order rate constants of Reactions 2 and 3 are not dependent on the enzyme concentration in the test range from 10 to 250 μM cytochrome aa_3.

The course of events emerging from the 605 nm observations was confirmed by tracing the 445 nm absorbance changes upon aeration of fully and partly reduced cytochrome aa_3. The identification of Reaction 3 as conversion of oxygenated to oxidized cytochrome aa_3 was facilitated by additional measurements at 578 nm.

The upward inflection of the first points in the experiment illustrated in Fig. 1, in which the reducing agent was dithionite, seems to suggest that the long aeration time masks a similar Reaction 2 as observed with NADH in Fig. 4. The zero time in Fig. 1 is rather arbitrarily fixed at the end of the 15-min aeration period.

Anaerobic titration with NADH

Since neither the $Na_2S_2O_4$-column procedure[13] nor use of H_2O_2 gave quantitative conversion of cytochrome aa_3 to the oxygenated enzyme, the classical $Na_2S_2O_4$/air procedure was used to prepare the oxygenated enzyme for the titration experiments.

Titration of oxidized cytochrome aa_3 under anaerobic conditions with NADH in the presence of a catalytic amount of phenazine methosulphate shows that 4 reducing equivalents are necessary for complete reduction of one enzyme molecule[1]. If the oxygenated compound were an oxygen or peroxide complex or a ferryl compound it would be expected to take up more than 4 electrons. When carrying out these titrations, the increasing amounts of NADH added to the Thunberg cuvettes containing oxidized cytochrome aa_3 are consumed within 2 min and the absorbance increase at 605 nm is then plotted against the number of reducing equivalents added. As preliminary experiments with oxygenated preparations indicated an unexpectedly slow reduction by NADH *plus* phenazine methosulphate, a different approach had to be used in this case.

Under strictly anaerobic conditions a 3-fold excess of NADH was added to the oxygenated enzyme in the presence of phenazine methosulphate and the absorbance at 605 nm was traced against the time (Fig. 5). The evacuation and temperature-

equilibration procedure causes a 14-min interval between the preparation of the oxygenated sample and the addition of NADH and phenazine methosulphate. Applying the rate constant for the decomposition of the oxygenated enzyme ($k = 4.5 \cdot 10^{-4}$ sec^{-1} at 22 °C), the percentage of the enzyme still oxygenated at the start of the reduction reaction may be calculated to be 68 %. This value agrees with the amount determined from the levels marked a, b and c in Fig. 5.

The distinctly biphasic reduction curve in Fig. 5 shows a rapid jump, followed by a very slow increase of the absorbance. The oxidized cytochrome aa_3 constituting 32 % of the starting mixture is rapidly reduced and the observed initial increase in 605 nm absorbance (0.688) corresponds closely to the calculated value (0.693) for the conversion of the oxidized fraction into the reduced form.

In addition to 605 nm the absorbance at 340 nm was recorded during the titration experiments by means of alternative scanning at the two wavelengths for short periods. This permits a direct correlation of the reduction of the enzyme with the oxidation of the NADH. As shown in Fig. 6 for the slow phase of the reduction, the plot of the absorbance at 340 nm against that at 605 nm gives a straight line. From the slope a millimolar absorbance coefficient (reduced *minus* oxygenated aa_3) of 26.6 $mM^{-1} \cdot cm^{-1}$ is calculated, which value has to be corrected for the small contribution of cyto-

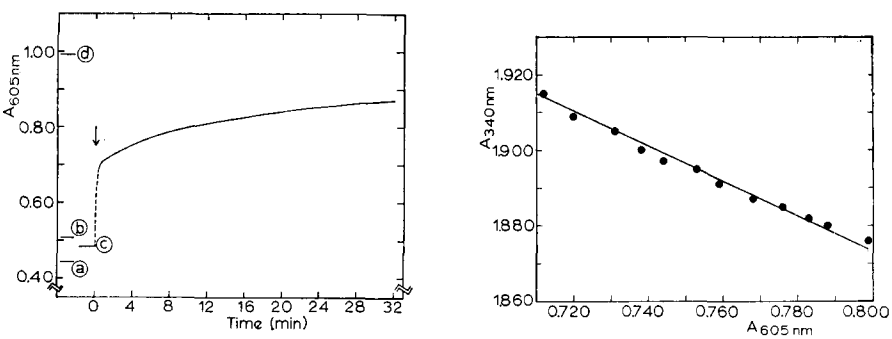


Fig. 5. Reduction of oxygenated cytochrome aa_3 with NADH and phenazine methosulphate under anaerobic conditions, followed at 605 nm. Oxygenated cytochrome aa_3 was prepared by the $Na_2S_2O_4$-air method. 22.8 μM cytochrome aa_3, 50 mM potassium phosphate (pH 7.2), 30 mM cholate, 137 μM NADH, 0.6 μM phenazine methosulphate, light path 1 cm, temperature 22 °C. The levels at the left give the absorbance at 605 nm of (a) oxidized aa_3, (b) oxygenated aa_3, (c) the mixture of oxidized and oxygenated cytochrome aa_3 when NADH *plus* phenazine methosulphate (at arrow) was added, (d) $Na_2S_2O_4$-reduced aa_3.

Fig. 6. The decrease of the absorbance at 340 nm plotted against the increase at 605 nm during the slow phase of the reduction in the experiment shown in Fig. 5.

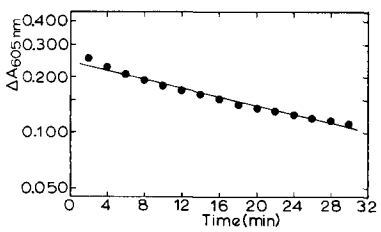


Fig. 7. Semilogarithmic plot of the slow phase of the reduction during the experiment shown in Fig. 5. $\Delta A_{605\ nm}$ is the difference between $A_{605\ nm}$ at time t and the final value.

chrome aa_3 to the absorbance difference at 340 nm. Using the ratio $A_{340\ nm}$ (reduced): $A_{340\ nm}$ (oxygenated) = 1.05 as deduced from other experiments, the true millimolar absorbance coefficient at 605 nm for cytochrome aa_3 (reduced *minus* oxygenated) becomes 20.8 $mM^{-1} \cdot cm^{-1}$. This value is based on the assumption that 2 moles of NADH (with 2×2 reducing equivalents) are consumed per mole cytochrome aa_3. The correctness of this assumption is proved by the fact that a millimolar absorbance coefficient (reduced *minus* oxygenated) of 21.0 is calculated directly from the difference spectrum (reduced *minus* oxygenated) of cytochrome aa_3.

The key for the reaction sequence during the slow phase of the titration is provided by the plot of log ($A_{605\ nm}$ at time t *minus* $A_{605\ nm}$ at time ∞) against the time in Fig. 7. From the resulting straight line a first-order rate constant of $4.5 \cdot 10^{-4}$ sec^{-1} is calculated, equal to that found for the normal decomposition of oxygenated cytochrome aa_3. This means that the oxygenated enzyme is not reduced at all by the NADH *plus* phenazine methosulphate system and is converted to the oxidized form at the same rate as under aerobic conditions. The oxidized enzyme, once formed, is rapidly reduced in a second reaction.

DISCUSSION

Decomposition of oxygenated aa_3

With a half-time of 20 min our oxygenated enzyme has about the same stability as that of others except for the most recent preparations of LEMBERG AND GILMOUR[24]. However, it is difficult to compare their values with our observations, because their half-time is not calculated from the decomposition kinetics studied at a fixed wavelength. LEMBERG and co-workers[24] use a peak wavelength scale to determine the percentage of oxygenated compound, in which 428 nm means 100 % oxygenated and 418 nm 100 % ferric oxidase.

During the decomposition reaction LEMBERG AND STANBURY[9] often observed a rapid shift to 424 nm followed by a very slow transformation to 418 nm if there was any further change at all. The γ-peak of our preparations reverted to the 422–424 nm value of the original oxidized enzyme. In agreement with others the cycle oxidized–reduced–oxygenated–oxidized never resulted in peaks below the starting wavelength.

The decomposition of oxygenated aa_3 follows first-order kinetics and has a normal Q_{10} value of 2.15, explaining the rapid decomposition observed by GILMOUR *et al.*[25] at 30°C. It has not been determined whether the observed rate constant is truly first order or only represents a pseudo first-order process involving water as a second reactant. The observation that the same value was found at pH 7.2 and pH 8.0 is not sufficient to rule out the possible intervention of H_3O^+ or OH^- ions.

The conversion of horseradish oxyperoxidase to ferric peroxidase also follows first-order kinetics as decribed by WITTENBERG and co-workers[10]. Although in this case the presence of a true oxygen-containing compound is likely, the pathway of the decomposition reaction is obscure. One of the oxidizing equivalents from the O_2 molecule is used for the oxidation of the ferrous iron; the fate of the remainder is unknown.

The decomposition rate of oxygenated aa_3 is the same under aerobic and anaerobic conditions, in contrast to the observations of LEMBERG AND GILMOUR[24] and DAVISON AND WAINIO[21], who found a strong acceleration of the reaction in the absence

of oxygen. The experimental conditions differ by the presence in our case of phenazine methosulphate and NADH.

Oxidation of cytochrome aa_3 after partial reduction with NADH

The formation of oxygenated cytochrome aa_3 after reduction of the oxidized enzyme with NADH has not been studied with a stopped-flow apparatus because it is almost impossible to work under truly anaerobic conditions without excess of reducing agent present. Thus neither the kinetics nor the first product of Reaction 1 have been recorded. We only know that at the end of the very rapid Reaction 1 the enzyme is largely present in the oxygenated form. From our experiments it cannot be decided whether the first product of the oxidation reaction is the ferric enzyme favoured by WHARTON AND GIBSON[26,27], or the oxygenated compound as argued by LEMBERG and his co-workers[25,28].

We found as the only condition for oxygenation by air, that at least one reducing equivalent must be present in the aa_3 molecule; the specific situation of this electron on the a_3 iron atom is not required (*cf.* ref. 13).

Titration with NADH

The anaerobic reaction of cytochrome *c* oxidase with NADH *plus* phenazine methosulphate is capable of differentiating kinetically between Fowler and Yonetani type of enzyme preparations (A.O. MUIJSERS, unpublished observations) in a similar way as found by GIBSON *et al.*[29,30] for the reduction by ferrocytochrome *c*. The non-reducibility of the oxygenated form must be due to some change in the enzyme itself since the intactness of both NADH and phenazine methosulphate is proved by the rapid reduction of the oxidized part of the enzyme sample.

From the titration it may be concluded that 4 reducing equivalents (or 2 NADH molecules) are consumed in the overall reaction from oxygenated *via* oxidized to reduced cytochrome aa_3. The known difference of 4 electrons between oxidized and reduced aa_3 (*cf.* ref. 1) suggests the same oxidation state for oxygenated and oxidized cytochrome aa_3. The same result was found by WILLIAMS *et al.*[13] by anaerobic reduction of the oxygenated compound by ferrocytochrome *c*. Though not mentioned by the authors, this redox equilibrium may also be reached by the same indirect way since the conversion of the oxygenated into the oxidized form is strongly accelerated by ferrocytochrome *c* (refs. 21,24).

The total of 4 oxidizing equivalents in the oxygenated enzyme argues against oxygen complexes but does not exclude the possibility of a different electron distribution among the 4 electron-accepting groups, for instance Fe(IV), Cu(I), Fe(III), Cu(II) (see ref. 14). The situation may be clarified by studying the 830 nm band. In the difference spectrum oxidized *minus* reduced aa_3 85 % of the absorbance is caused by one Cu-atom and 15 % by the second[31], in contrast to earlier conclusions[1]. The 830-nm band of the oxygenated compound, however, is equal to that of the oxidized enzyme[32] or is even a little higher[8]. The possibility of a compensation of the lost 830 nm absorbance of one copper atom by a new infrared band of the ferryl iron of a_3 at about the same wavelength is an unlikely coincidence. Our results, however, are not in conflict with LEMBERG's suggestion about compensation of the ferryl iron by an unknown reduced group (called XH_2 or XH) somewhere in the protein[14].

As shown by us in anaerobic titration experiments of the oxidized enzyme with

NADH, cytochrome *a* and one copper can be reduced under conditions in which the reduction of a_3 is blocked by the presence of azide or cyanide[1]. The non-reducibility with NADH and PMS of the whole oxygenated molecule does not support the conclusion of WILLIAMS *et al.*[13] that only a_3 is affected. The behaviour of the oxygenated enzyme during titration might be connected with (or forms an extreme example of) the lag in the reduction of this compound as observed by LEMBERG AND GILMOUR[24] with dithionite and by WILLIAMS *et al.*[13] with *N,N,N',N'*-tetramethyl-*p*-phenylenediamine or with ascorbate *plus* a trace of cytochrome *c*. From the work of WILLIAMS *et al.*[13] and LEMBERG AND CUTLER[33] it might be concluded that this lag represents a conversion of a_{3X}^{4+} to a_{3X}^{3+} which is rapidly reduced to a_3^{2+} with the same velocity as reduction of a^{3+}. In the experiments of LEMBERG AND CUTLER[33] on reduction of oxygenated cytochrome aa_3 with stoicheiometric amounts of ferrocytochrome *c* no lag is mentioned and the conversion of the ferryl form seems to be strongly accelerated under these conditions.

An oxygenated form of cytochrome P-450 has recently been discovered by ISHIMURA *et al.*[11] and by ESTABROOK and co-workers[34]. This compound has some common characteristics with oxygenated cytochrome aa_3 but not enough to substantiate a claim for the existence of a cytochrome aa_3-oxygen complex.

The properties of horseradish peroxidase Compound III also show similarity with "oxygenated" cytochrome aa_3, but the Fe (IV)O_2^- structure preferred by YAMAZAKI *et al.*[35] should give rise to an extra consumption of NADH in our anaerobic titration. For oxyperoxidase, which appears to be identical with Compound III (ref. 10), the structure Fe(III)O_2^- was proposed by WITTENBERG[36]. Whether or not the above mentioned compounds really contain oxygen, the simultaneous recording of enzyme reduction and NADH oxidation during our titration experiments with "oxygenated" cytochrome aa_3 leaves no room for extra oxidizing equivalents. It should be noted that in this titration with NADH and phenazine methosulphate H_2O_2, the superoxide anion, as well as molecular oxygen (set free by dissociation of an oxygenated complex) would surely be detected by direct reaction with reduced phenazine methosulphate even if the cytochrome aa_3 present did not react with it. It is concluded that "oxygenated" cytochrome aa_3 is not a true oxygenated complex and also not of peroxidic nature. The proposed structure with one ferryl iron and one monovalent copper[14] is left open by the titration experiments but is not supported by the study of the 830-nm band. From the remaining possibilities suggested by LEMBERG[14] and by WHARTON AND GIBSON[26] the idea of oxygenated cytochrome aa_3 as different conformation of the oxidized enzyme appears to be the most likely one.

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