BBA 45594

CYTOCHROME OXIDASE AND ITS DERIVATIVES

VIII. FORMATION AND PROPERTIES OF THE 'OXYGENATED' FORM OF CYTOCHROME OXIDASE AND ITS RECONVERSION TO FERROUS AND FERRIC OXIDASE

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(Received May 8th, 1967)

SUMMARY

1. The 'oxygenated' compound of cytochrome *c* oxidase used in our experiments is more stable than the compound of previous reports. It is quantitatively reversible to ferrous oxidase.

2. It is best formed with an excess of O_2 after reduction with a minimum amount of dithionite. It can also be formed at low O_2 tension, but then contains some ferric oxidase.

3. Its formation from ferrocyanide-reduced oxidase remains incomplete and subsequent reduction by dithionite is also incomplete.

4. Cyanide does not inhibit its formation from ferrous oxidase. If only ferricytochrome *a* but no ferricytochrome a_3 is reduced in the presence of cyanide by dithionite, there is no reaction with O_2.

5. The anaerobic reduction of 'oxygenated' oxidase by dithionite is monophasic and fast. In contrast, that of ferric oxidase is biphasic, with an initial fast reduction of ferricytochrome *a* followed by a much slower reduction of ferricytochrome a_3. The rate of cytochrome *a*, but not that of cytochrome a_3 reduction depends on dithionite concentration.

6. In the presence of dissolved O_2, the ferric oxidase reduction comes to a temporary standstill when one-third of the absorbance increase at 444 mμ has been reached.

7. Ethyl hydrogen peroxide reacting with ferrous oxidase forms a compound similar to the 'oxygenated' compound.

8. Hydrogen donors known to react with peroxidase–H_2O_2 complexes, particularly pyrogallol, accelerate the transformation of 'oxygenated' to ferric oxidase, though not at a rate comparable to that of cytochrome *c*.

9. These results strengthen the evidence for cytochromes *a* and a_3 but indicate that this difference has disappeared in 'oxygenated' oxidase.

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INTRODUCTION

The reaction of ferrous cytochrome *c* oxidase (EC 1.9.3.1) with molecular oxygen has been studied previously by ORII AND OKUNUKI[1–4], by DAVISON AND WAINIO[5,6] and by LEMBERG and co-workers[7,8]. Differences in the rate of dithionite reduction of ferric and of 'oxygenated'* oxidase were noted by WAINIO (ref. 6, p. 633) and by OKUNUKI[9], while LEMBERG *et al.*[10] and LEMBERG AND MANSLEY[11] have shown that ferricytochrome *a* is reduced much faster by dithionite than ferricytochrome a_3. This difference has, however, not been observed with NADH–phenazine methosulphate[12].

The present paper shows essential differences in the kinetics of the reduction of oxygenated and ferric oxidase by dithionite.

It has been shown that the oxygenated oxidase is not a mixture of ferric with ferrous oxidase[1–8]; that it is formed by direct reaction of ferrous oxidase with molecular oxygen, but is not a reversibly oxygenated compound similar to oxyhaemoglobin[7,8], as was assumed by ORII AND OKUNUKI[1,2,4] and WAINIO[5,6], that it is readily formed by the action of H_2O_2 on ferrous oxidase (LEMBERG and co-workers[7,8]; but see ORII AND OKUNUKI[3,4]). These findings indicate that the oxygenated oxidase is of peroxidic nature. Observations of YAMAZAKI AND PIETTE (1963)[13] and YAMAZAKI AND YOKOTA (1965)[24] on compounds formed from horse radish peroxidase (*e.g.* compound III) indicate that peroxidase 'compounds III' are close to oxygenated oxidase in their mode of formation and in their properties. They too are not reversible ferrous–oxygen complexes of the oxymyoglobin type.

We have therefore studied the reaction of oxygenated oxidase with hydrogen donors which are known to accelerate the conversion of complex III to ferric peroxidase[25], as well as the ethyl hydrogen peroxide compound of cytochrome oxidase and the effect of cytochrome *c* peroxidase on the oxygenated oxidase.

MATERIALS AND METHODS

Cytochrome c oxidase was prepared by the modified Okunuki–Yonetani method as previously described[11,14] except that a solution of Tween-80 (polyoxysorbitane monooleate, Sigma) was used instead of Emasol. No dithionite was added at any stage of this preparation. The ratio $A_{280\,m\mu}$ $Fe^{3+}/A_{444\,m\mu}$ Fe^{2+} was 2.3–2.8. For all assays the oxidase was diluted with 0.1 M phosphate (pH 7.4) containing 0.5 % Tween-80. The oxidase concentration is expressed as μM haem *a* on the basis of ε_{mM} at 605 mμ = 23 for ferrous oxidase.

Absorption spectra and kinetics of alteration of absorbance were measured on a Cary-15 recording spectrophotometer either in evacuated optical Thunberg tubes ('anaerobic') or in optical cuvettes covered by a coverslip ('aerobic'). In the reduction experiments the time difference between the admixing of dithionite and the first recorded absorbance in the spectrophotometer was measured by stopwatch (usually 10–15 sec) and this was added to the time measured on the chart involving a movement of 13 sec or 50 sec per division. In the anaerobic experiments 3 ml of the solution was placed in the optical part of the Thunberg tube, and solid sodium dithionite, 100 times diluted by sucrose, in the hollow stopper[14]. The tube was then closed and evacuated

* For simplicity's sake the inverted commas, indicating that 'oxygenated' oxidase is not a reversibly oxygenated compound like oxyhaemoglobin will be omitted below.

to about 5 mm mercury by a high vacuum oil pump. Any changes of volume due to evaporation during this procedure were corrected.

Figs. 4 and 6 were obtained with a Perkin–Elmer recording spectrophotometer with the technical assistance of Miss M. GRAY (Institute of Medical Research, Royal North Shore Hospital).

RESULTS

The oxygenated compound of cytochrome oxidase

Fig. 1 shows the spectrum of the oxygenated compound of cytochrome oxidase formed using optimal conditions compared with spectra of the ferric and ferrous forms. The ferric oxidase was reduced under anaerobic conditions (see MATERIALS AND METHODS) with a minimal amount of dithionite and the oxygenated form subsequently produced by rapidly bubbling O_2 gas through the ferrous enzyme solution. Isosbestic points are found between the ferrous and ferric $(a + a_3)$ curves at 620, 558, 462 and 432.5 mμ, between the ferrous and oxygenated curves at 620, 584, 461 and 435 mμ, and between the ferric and oxygenated curves at 620, 484–511 (flat) and 424 mμ.

The oxygenated form of cytochrome oxidase can be reduced with dithionite under anaerobic conditions producing ferrous oxidase with a spectrum identical to that found before the oxygenation, and on reoxygenation a spectrum identical with the original oxygenated form is found. Correction was made for changes in concentration due to evaporation on evacuation. This process of oxygenation and back reduction can be repeated several times with no alteration of either the oxygenated or ferrous

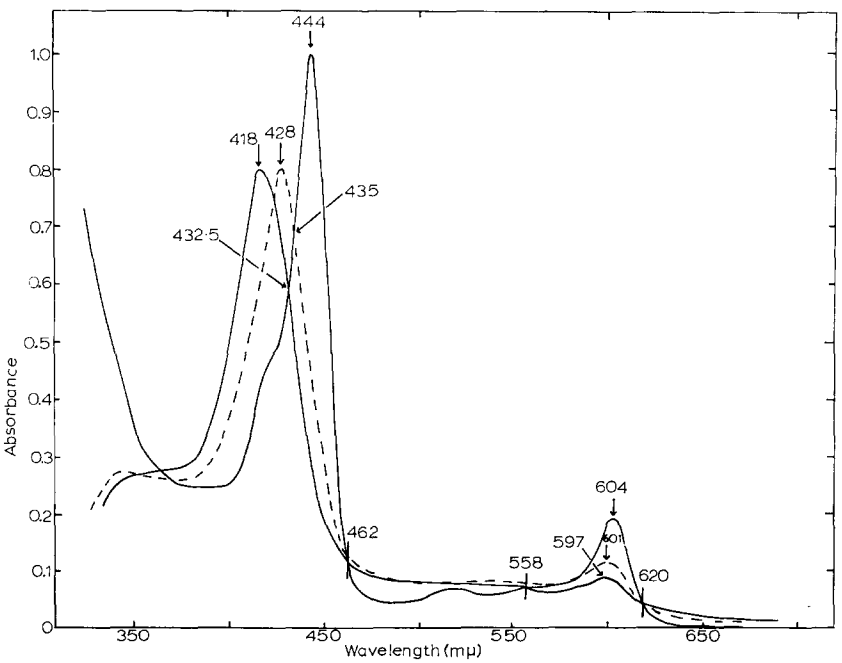

Fig. 1. Absorption spectra of ferrous (max. 604 and 444 mμ), ferric (max. 597 and 418 mμ), and oxygenated (broken line, max. 601 and 428 mμ) cytochrome oxidase.

spectra. Experiments in which the oxygenated compound was produced by H_2O_2 showed a variable destruction (up to 20 %) of the enzyme.

Temporary formation of the oxygenated compound in the presence of excess dithionite

Cytochrome oxidase was reduced in the presence of excess dithionite and small amounts of air were subsequently bubbled through the solution (Fig. 2). There was a time lapse of approx. 1 min from the recording of the α-peak to that of the Soret peak.

The curves show that in the presence of excess dithionite ferrous oxidase rapidly reacts with O_2 and this oxygenation is subsequently followed by reduction by dithionite back to the ferrous form.

All curves pass through an isosbestic point at 433 mμ which was the position reported by LEMBERG AND STANBURY[8]. This is in contrast to the isosbestic point at 435 mμ when the oxygenated compound was formed using optimal conditions. This difference may indicate that the point where all the curves cross is not a true isosbestic point between two components (ferrous and oxygenated forms) but that the reaction involves formation of some ferric oxidase in constant proportion to that of the oxygenated oxidase. This is supported by the position of the Soret maximum of the finally formed oxygenated compound at 425 mμ compared with the 428 mμ maximum of the oxygenated compound of Fig. 1.

Formation of the oxygenated compound at low O_2 tension

If, instead of O_2 gas or air, N_2 gas containing approx. 0.5 % O_2 was bubbled through a solution of ferrous oxidase (previously produced under anaerobic conditions

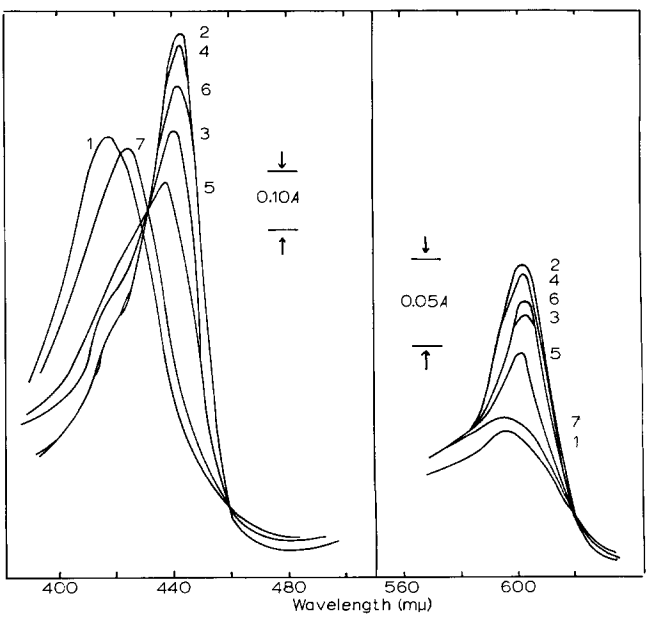

Fig. 2. Temporary formation of the oxygenated compound in the presence of excess dithionite. 8.4 μM oxidase (J-1). Curve 1, ferric oxidase; Curve 2, ferrous oxidase after 30 min reduction with 0.094 mg dithionite (100 times diluted with solid sucrose) in 3 ml. All times stated are after the first oxygenation; Curve 3, 36 sec after partial oxygenation; Curve 4, 4 min 15 sec, standing; Curve 5, 15 min, after further oxygenation; Curve 6, 18 min, standing; Curve 7, 32 min, complete oxygenation.

with a minimal amount of dithionite) the ferrous was slowly converted to the oxygenated form as shown in Fig. 3. Most residual dithionite was oxidized during the first few minutes of N_2 bubbling, before recording of Curve 3, as can be seen by the decrease in absorbance at 330 mμ. Subsequent recordings show only a small additional decrease. All curves intersect at a point close to 433 mμ and the peak position of the fully formed oxygenated compound is at 426 mμ similar to that found in Fig. 2. The formation of the oxygenated compound at this low O_2 tension is slow (incomplete even after 30 min) compared with the almost instantaneous formation observed at atmospheric O_2 tension. Although the reaction is slow the results show that the oxygenated compound can be formed at very low O_2 tension.

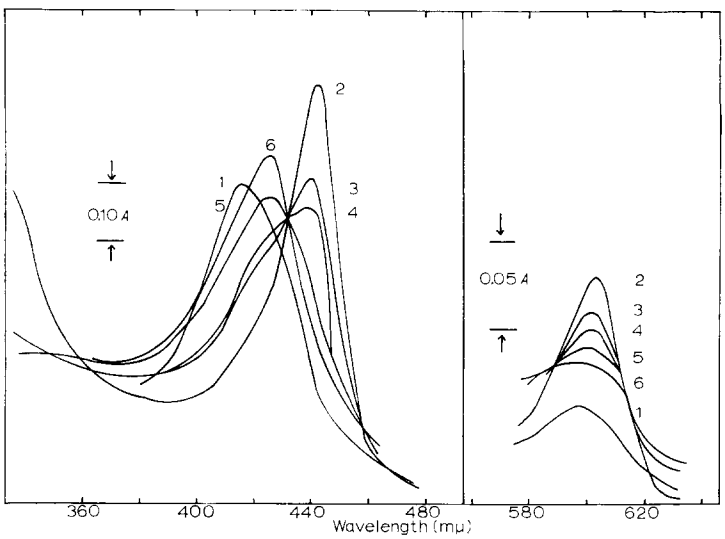

Fig. 3. Formation of oxygenated oxidase at low oxygen tension. 6.4 μM oxidase (J-1). Curve 1, ferric oxidase; Curve 2, ferrous oxidase after 30 min reduction by 0.085 mg dithionite (100 times diluted with solid sucrose) in 3 ml; Curves 3–5, bubbling through N_2 with 0.5% (v/v) of O_2; Curve 3, 7 min; 4, 10 min; 5, 30 min; Curve 6, after oxygenation with O_2.

Formation of an oxygenated-like compound with ethyl hydrogen peroxide

Anaerobic addition of ethyl hydrogen peroxide to ferrous cytochrome oxidase formed a compound whose spectrum resembled that of the oxygenated form. Ferrous oxidase (7.7 μM) was produced by reduction of the ferric form with dithionite (100 μM) in an evacuated Thunberg cell, then 0.6 mM ethyl hydrogen peroxide was added through the side arm to a final concentration of 200 μM and the spectrum recorded immediately. The Soret maximum is at 425 mμ and the α-maximum at 598 mμ. The isosbestic point between the ferrous and the ethyl hydrogen peroxide curves in the Soret region lies at 433 mμ. The Soret peak height is 0.93 that of the ferric peak absorbance. The ratios of the peak absorbances of the ferrous form to the ethyl hydrogen peroxide compound, 444 to 425 mμ and 604 to 598 mμ, are 1.35 and 1.66, respectively.

In contrast to the formation of the oxygenated compound with H_2O_2 (*cf.* ref. 8) a greater excess of ethyl hydrogen peroxide was required for complete conversion of the ferrous form. Due to the presence of residual dithionite after oxidase re-

duction, the proportion of oxidant required was expressed as moles of oxidant per mole of dithionite. This ratio was less than 1 for the H_2O_2 effect, but varied from 1.6–2.6 for the ethyl hydrogen peroxide. Ethyl hydrogen peroxide had no effect when added in the same concentration to the ferric enzyme.

Formation of oxygenated oxidase following ferrocyanide reduction

Ferric oxidase is slowly reduced by $K_4Fe(CN)_6$ as shown in Fig. 4. The ferrocyanide solution was added anaerobically (through the side arm of the Thunberg tube) to the ferric enzyme solution in its main cuvette, after some dithionite had been placed into the hollow stopper and the tube had been evacuated to ensure complete

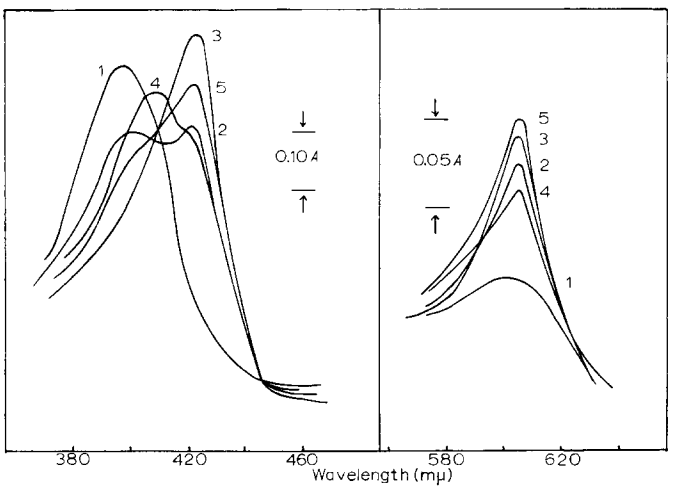

Fig. 4. Formation of oxygenated oxidase following anaerobic reduction by potassium ferrocyanide. 12.6 μM oxidase (G-1). Curve 1, ferric oxidase, corrected for subsequent dilution with ferrocyanide; Curve 2, 1 min after ferrocyanide addition to a final concn. of 3.3 mM; Curve 3, after 165 min; Curve 4, after aeration; Curve 5, after 18 min reduction with excess dithionite.

anaerobiosis. The spectrum recorded immediately after ferrocyanide addition showed about 50 % reduction of the Soret band, but almost complete reduction of the α-band. After 165 min, reduction of the Soret band was 75 % complete. At this stage the tube was opened and vigorously aerated. This produced the formation of a Soret maximum at 428 mμ (Curve 4). However, the oxygenated oxidase still contained some ferrous oxidase, 35 % as calculated from the shoulder at 444 mμ. Further oxygenation did not abolish this shoulder. The subsequent addition of dithionite (Curve 5) reduced the enzyme only to the extent of 65 %. Thus it appears that both the oxygenation of the ferrous enzyme and the reduction of the oxygenated compound are partially inhibited. This inhibition is probably due to the presence of cyanide ion, formed by partial dissociation of the ferrocyanide complex.

If reduction by ferrocyanide was carried out in an evacuated Thunberg cell without additional provision for removing residual O_2, the curves of Fig. 5 were obtained. Initially the 420 mμ maximum decreased and reduction continued to about 50 %, while simultaneously the Soret maximum shifted to 425 mμ in 15 min and to 429 mμ in 60 min. After opening the tube and aeration, the Soret maximum was at

427 mμ, the usual position for oxygenated oxidase. However, while the absorbance at 444 mμ decreased it remained higher than that of typical oxygenated oxidase. Addition of dithionite caused only 80 % reduction.

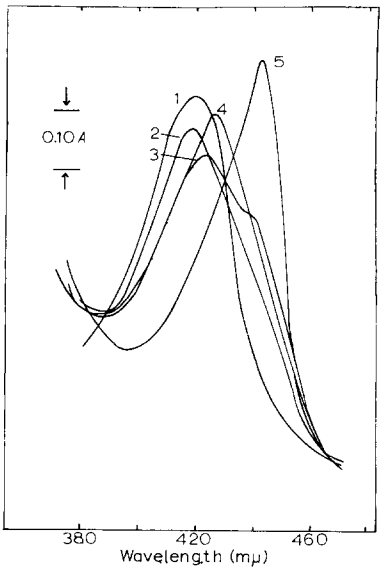

Fig. 5. Formation of oxygenated oxidase following reduction by $K_4Fe(CN)_6$ with incomplete removal of air. 10.5 μM oxidase (J-3). Curve 1, ferric oxidase, corrected for subsequent dilution with ferrocyanide; Curves 2 and 3, 1 min and 60 min after ferrocyanide addition to a concentration of 4 mM; Curve 4, after aeration; Curve 5, after 15 min reduction with excess dithionite.

In an experiment in which 8 μM oxidase was first formed and then exposed to 3.2 mM ferrocyanide in an open cuvette, only small spectral alterations occurred, a small increase in the α-band and a 2 mμ shift of its maximum to longer wavelengths, a small decrease in the Soret band absorption with an 1.5 mμ shift towards shorter wavelengths, and an increase in the absorption at 444 mμ. Reduction by excess dithionite (10 min) measured at 444 mμ was only 82 %, at 605 mμ 94 %. Similar small spectral alterations were observed when NaCN, instead of ferrocyanide, was added to oxygenated oxidase.

Formation of the oxygenated compound in the presence of cyanide

Addition of NaCN to a final concentration of 3.2 mM converted the absorption spectrum of 7.4 μM ferrous cytochrome oxidase into the well-known absorption spectrum of the ferrous cyanide oxidase with its maximum at 601 mμ with a broad shoulder at 589 mμ and its decreased Soret band at 443 mμ. Aeration rapidly converted the spectrum into that of the oxygenated oxidase with its Soret maximum at 428 mμ and its absorbance 0.975 that of the original ferric absorbance at 418 mμ. The only difference of this spectrum from that of the oxygenated oxidase obtained in the absence of cyanide was its slightly higher absorption at 601 mμ and at 443 mμ (about 12 %) which may indicate the presence of some ferrous cyanide oxidase. If one calculates the difference spectrum from the ferric oxidase to this oxygenated compound the maximum absorbance increase is found not at 428 mμ but at 442 mμ. In spite of

this evidence for incomplete oxygenation subsequent addition of excess dithionite restored only 84 % of the fully reduced 443 mμ absorbance. In contrast the α-band showed complete reduction.

When ferric oxidase was reduced by dithionite in the presence of cyanide and subsequently aerated, a similar effect was obtained. To an 8-μM solution of ferric oxidase containing 3.4 mM cyanide in one evacuated Thunberg cell dithionite was added to a 100 μM concentration. 40 min was allowed for reduction. Calculated from the absorbance change at 444 mμ the enzyme was 66 % reduced. Since the contribution of cytochrome *a* is certainly less than 50 % some ferricytochrome a_3–cyanide complex had, under these conditions, undergone reduction to ferrocytochrome a_3–cyanide (*cf.* ref. 10). Vigorous aeration caused a slow formation of the oxygenated compound. This formation was still quite incomplete 15 min after aeration, but it reached completion by the 45 min recording of the spectrum as indicated by a Soret maximum at 428 mμ with an absorbance equal to that of the original ferric oxidase at 418 mμ. As above, the absorbances at 601 mμ and particularly at 444 mμ were higher than for oxygenated oxidase formed in the absence of cyanide. Reduction with excess dithionite for 10 min gave essentially the same absorption spectrum as that before aeration.

If dithionite reduction of cytochrome oxidase in the presence of cyanide is terminated by oxygenation when only ferricytochrome *a*, but no significant amount of ferricytochrome a_3–cyanide complex has been reduced, oxygenation causes essentially no alteration of the partially reduced spectrum. This is shown in Fig. 6. Two min after mixing dithionite with ferric oxidase in an evacuated Thunberg cell the spectrum (Curve 2) was recorded. The absorbance increases at 605 and 443 mμ represent 79 and 47 %, respectively, of that for a fully reduced enzyme ($a + a_3$) which are roughly the absorbance contributions of ferrocytochrome *a*. The solution was then (about 5 min after the dithionite addition) vigorously bubbled with O_2 gas. The spectrum (Curve 3) shows essentially no decrease at 443 mμ or increase at 428 mμ to indicate formation of oxygenated compound. Thus ferrocytochrome *a* alone does not react with O_2.

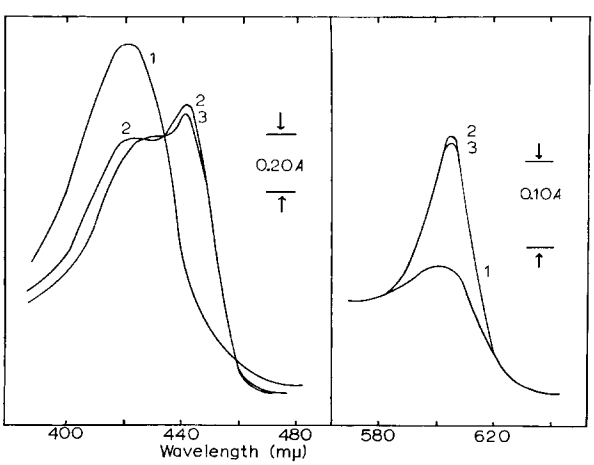

Fig. 6. Effect of aeration on partially reduced cytochrome oxidase ($a^{2+} + a_3^{3+}$ CN). 12.6 μM oxidase (G-1), 3.4 mM cyanide. Curve 1, ferric cyanide oxidase; Curve 2, after 2 min reduction with 0.042 mg dithionite (diluted 100 times with solid sucrose) in 3 ml; Curve 3, aerated for 3 min.

Upon standing for about 30 min there is a small increase in absorbance at 433 mμ indicating a very partial formation of oxygenated compound.

Dithionite reduction of ferric ($a + a_3$) and oxygenated oxidase

Curve A of Fig. 7 shows the absorbance change at 444 mμ as a function of time when ferric cytochrome oxidase is reduced by dithionite in an evacuated Thunberg cell. The curve has a biphasic character with an initial rapid absorbance increase to somewhat less than 50 % of the total change followed by a much slower increase. LEMBERG AND MANSLEY[11] have shown previously that cytochromes a and a_3 have different rates of dithionite reduction, and that the γ/α ratio of the rapidly reduced ferric component is that of cytochrome a and the ratio of the slowly reduced component that of cytochrome a_3. In Curve B, where approx. 50 times less dithionite was used, the initial part of the reaction is considerably slower than in Curve A, whereas the final phases are nearly parallel, showing a dithionite dependence for cytochrome a reduction but a virtual independence for cytochrome a_3 reduction.

When dithionite reduction of the oxygenated form of cytochrome oxidase was studied as above, the curve (Curve C) is quite different. The absorbance increase is monophasic and rapid. In addition, the reduction of the oxygenated compound is more dependent on dithionite concentration (see Curves C and D) than that of ferricytochrome a (initial part of Curves A and B). The rate of the initial reduction of oxygenated compound was increased approx. 10-fold by a 48-fold increase in dithionite concentration whereas the initial reduction rate of ferricytochrome a was increased only 4-fold.

With a 4750-μM concentration of dithionite the initial rate of reduction of oxygenated oxidase equals that of the reduction of ferric cytochrome a, but with 100 μM dithionite it is somewhat slower.

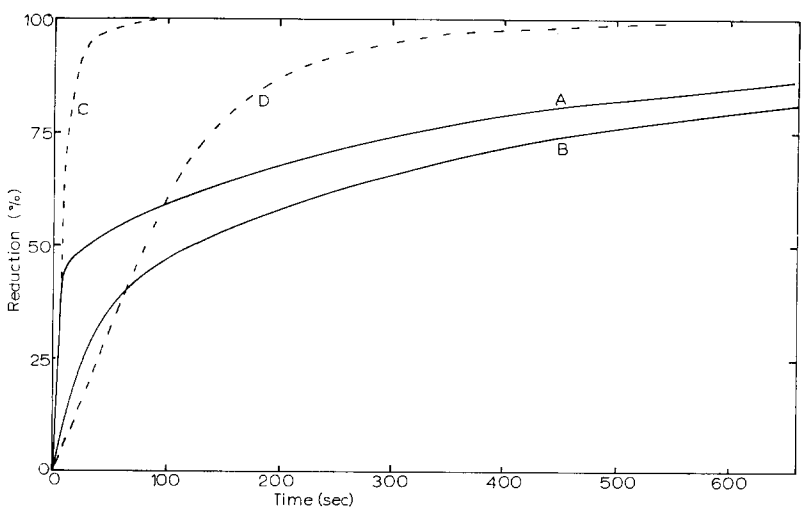

Fig. 7. Dithionite reduction (anaerobic) of ferric and of oxygenated cytochrome oxidase. Increase of $A_{444\ m\mu}$ on reduction of ferric cytochrome oxidase ($a + a_3$) (8 μM for haem a). Curve A, by 4.75 mM dithionite; Curve B, same with 100 μM dithionite; Curve C, increase of $A_{444\ m\mu}$ on reduction of oxygenated cytochrome c oxidase by 4.75 mM dithionite; Curve D, same with 100 μM dithionite. All expressed as percentages of maximal rise on complete reduction.

Calculation of the half-times for ferric oxidase reduction is complicated by the different rates of reduction of the *a* and a_3 compounds; in calculating them we have assumed that cytochrome *a* reduction is essentially complete before a_3 reduction begins. Relative absorbance contributions of ferrous *a* and a_3 at 444 mμ are disputed. The half-time values which are given in Table I have been calculated using two different assumptions: (a) that cytochrome *a* contributes 50 % of the total absorbance (Fe^{2+}—Fe^{3+}) according to YONETANI[15] and VAN GELDER[12] or (b) that it contributes only 33.6 % according to VANNESTE[16]. The actual half-times observed for reduction of ferricytochrome *a* are in good agreement with those calculated from the intitial linear part of the curves particularly if a 50 % contribution of ferrocytochrome *a* is assumed. The average half-time for ferricytochrome *a* reduction by 100 μM dithionite is 21 sec assuming a 50 % contribution, 13 sec assuming a 33.6 % contribution, whereas the corresponding half-times for ferricytochrome a_3 reduction are 10 to 16 times as long. At high concentrations of dithionite this ratio increases to about 65.

TABLE I

ANAEROBIC REDUCTION OF FERRICYTOCHROMES *a* AND a_3 BY DITHIONITE MEASURED AT 443 mμ

Dithionite (μM)	*Half-time of reduction* (*sec*)					
	Assuming a 50 % contribution by a			*Assuming a 33 % contribution by a*		
	a^*	a^{**}	$a_3{}^{**}$	a^*	a^{**}	$a_3{}^{**}$
80	21.2	20.0	200	14.3	10.5	175
100	16.2	20.2	250	10.9	13	200
133	25.2	24.0	227	17.0	15.8	184
4750	5.15	4.3	293	3.45	2.9	181

* Calculated from initial rate of increase.
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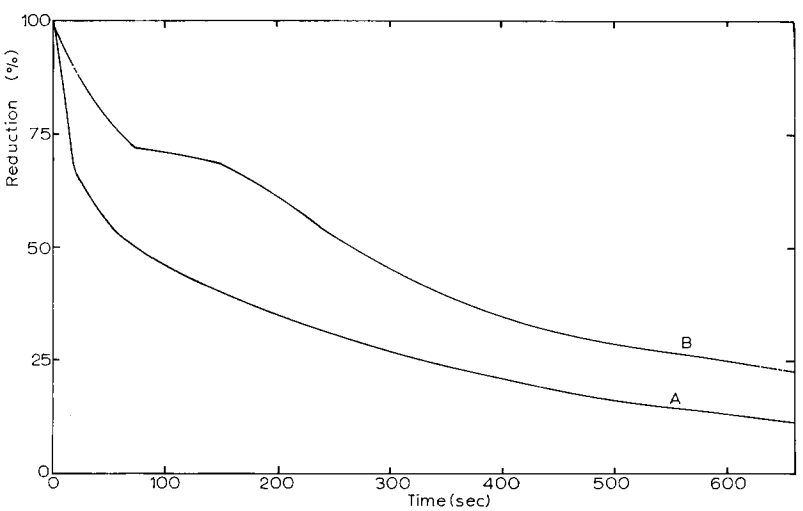

Fig. 8. Dithionite reduction of ferric cytochrome oxidase measured by the decrease of $A_{419\ m\mu}$. Curve A, anaerobic reduction by 4.7 mM dithionite; Curve B, aerobic reduction by 0.54 mM dithionite.

The biphasic dithionite reduction of the ferric enzyme is also observed (Curve A, Fig. 8) when the decrease in absorbance at the ferric Soret maximum, 419 mμ, is measured. At 605 mμ, where cytochrome *a* contributes between 81–85 % of the differential absorption, the slow phase of the cytochrome a_3 reduction is still distinguishable though, of course, less marked (see Fig. 9).

An attempt was made to correlate the absorbance increase at 605 mμ with that at 444 mμ in order to determine the relative contribution of ferrocytochromes *a* and a_3 to the 444 mμ absorption. The results are in better agreement with a 35 %

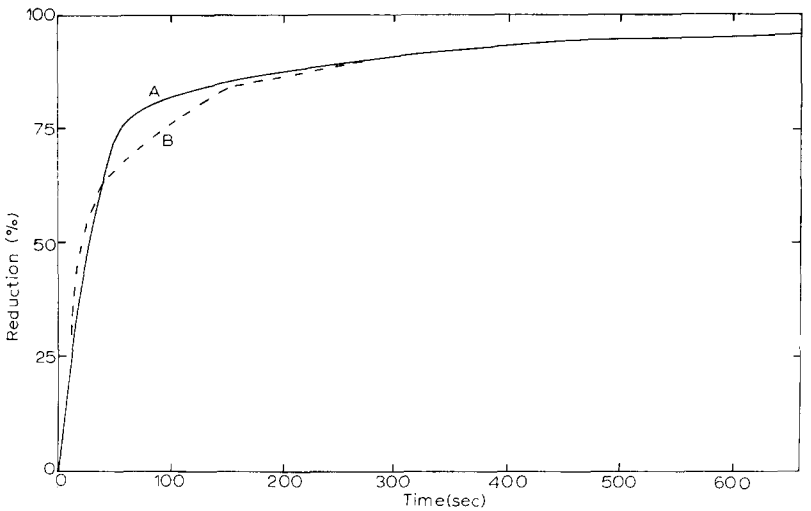

Fig. 9. Dithionite reduction of ferric cytochrome oxidase measured by the increase of $A_{605\ m\mu}$. Curve A, anaerobic reduction by 100 μM dithionite; Curve B, aerobic reduction by 540 μM dithionite.

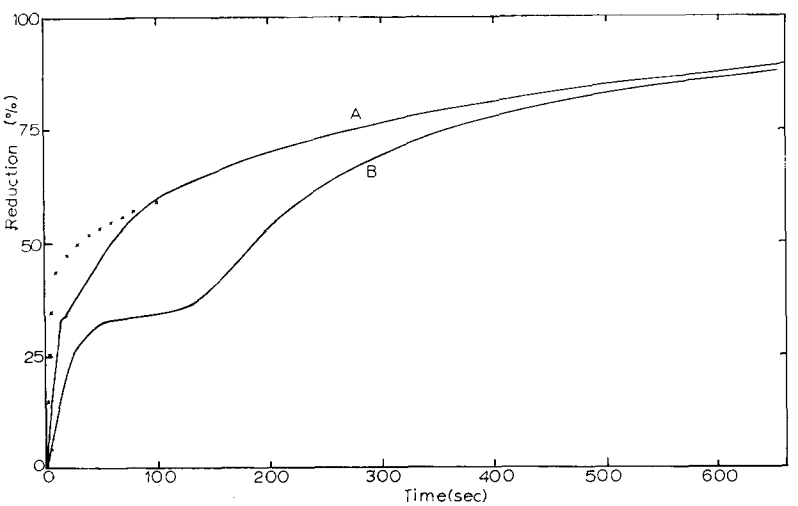

Fig. 10. Dithionite reduction (aerobic) of ferric cytochrome oxidase. Increase of $A_{444\ m\mu}$ in percent of final. Curve A, by 4.75 mM dithionite; the crosses indicated corresponding values in anaerobic reduction; Curve B, by 540 μM dithionite.

than with a 50 % contribution of ferrocytochrome *a* to the absorbance at 444 mμ, but, for various reasons, cannot as yet be considered decisive.

Dithionite reduction of ferricytochrome oxidase in the presence of air

When dithionite is added to an aerated solution of ferric cytochrome oxidase in a cuvette closed only by a cover-slip and the absorbance change observed at 444 mμ, an interesting phenomenon occurs as shown by the curves in Fig. 10. The absorbance increases almost linearly to approx. one-third of the total reduction, then for a variable time interval it levels off with no further increase, after which the absorbance increase resumes but at a slower and, for a short time, almost linear rate. The duration of the stationary phase after about 30 % reduction is inversely dependent on the dithionite concentration; it is caused by the presence of O_2 and reduction resumes only after all O_2 in the solution has been reduced. If it is tentatively assumed that ferrocytochrome *a* is reduced by dithionite before a_3 reduction begins and that ferrous *a* cannot react directly with O_2, then the initial fast absorbance increase can be assigned to cytochrome *a* reduction. The stationary phase at approximately the 30 % level of reduction would signify the end of *a* reduction and the linear rise afterwards the beginning of a_3 reduction. In Table II the half-times of reduction of ferricytochrome *a* are calculated from the initial linear part of the curve (Column 3) and from the observed half-point between the base and the stationary phase (Column 4). The half-times of reduction of ferrocytochrome a_3 are calculated from the linear part of the curve immediately after the stationary phase (Column 5) and from the observed half-point between stationary phase level and 100 % reduction (Column 6). Good agreement exists between the half-time values computed by the two methods; there is, however, considerable variation between different experiments, particularly for cytochrome a_3. A comparison of these values with equivalent ones in Table I shows that the cytochrome *a* reduction is slower in the presence of air, but that despite a 5-fold increase in dithionite concentration the rate of cytochrome a_3 reaction is about the same.

TABLE II

REDUCTION OF FERRICYTOCHROMES *a* AND a_3 BY DITHIONITE IN SOLUTION CONTAINING AIR MEASURED AT 443 mμ

Dithionite (μ*M*)	*Level of stationary phase as percentage of total increase*	*Half-time of reduction in sec assuming a 33 % contribution of a*					
		a^*	a^{**}	a_3^{***}	a_3^{**}	*Relative velocities*	
						V_{a/a_3}§	V_{a/a_3}§§
400	32	24.5	25	205	286	8.4	11.4
540	34	13.1	13.7	122	211	9.3	15.4
540	28	30	30	135	185	4.5	6.2
540	28.5	62	71	383	350	6.2	4.2
540	31.5	19.5	21.5	133	130	6.8	6.0
4700	32.5	8	6.8	108	143	13.5	21

* Calculated from initial rate of increase.
** Found.
*** Calculated from linear rate after stationary phase.
§ Calculated from a^* and a^{***}.
§§ Calculated from half-times.

Although the rapidity of the absorbance increase on reduction at 605 mμ makes exact determinations difficult, preliminary experiments indicate a similar, but less distinct, standstill at about the 75 % level of reduction. This is somewhat lower than the 81–85 % absorbance contribution found for cytochrome *a*.

NADH–phenazine methosulphate reduction of ferricytochrome oxidase

The reduction of cytochrome oxidase by NADH–phenazine methosulphate in an evacuated Thunberg cell as observed at 444 mμ is shown in Fig. 11. The curve shows that the reduction of the ferric enzyme by this reductant is kinetically quite different from the dithionite reduction. There is not the sharp differentiation between the reduction rates of cytochrome *a* and a_3 although there is a small linear final portion in the curve involving the last 10–15 % of the total reduction. These results are in excellent agreement with those of VAN GELDER (ref. 12, Fig. 1).

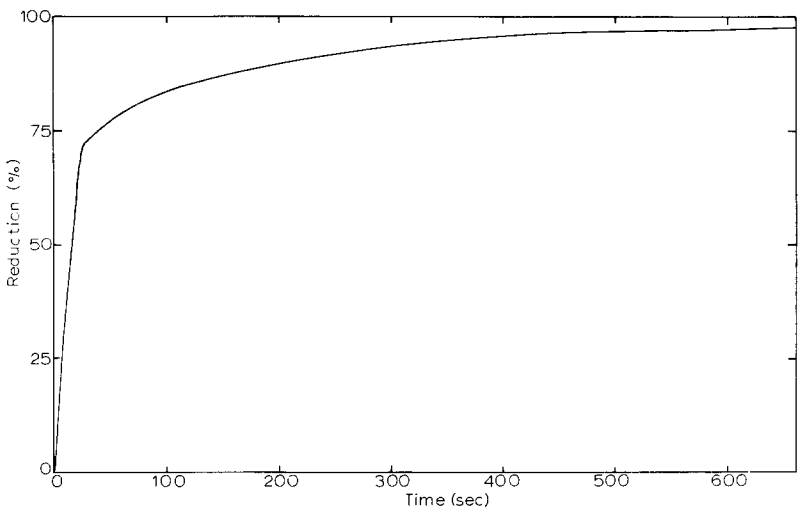

Fig. 11. Reduction (anaerobic) of ferric cytochrome oxidase by NADH–phenazine methosulphate. Increase of $A_{444\ m\mu}$ in percent of final by 13 μM NADH (as disodium salt) and 5.7 μM phenazine methosulphate added to ferric cytochrome oxidase (6.5 μM).

Transformation of oxygenated into ferric oxidase by hydrogen and electron donors

With the preparations now used the oxygenated compound has been found to be very stable. Compared with the earlier reports of 5–20 min for the half-time of the spontaneous shift of the 428 mμ oxygenated peak to the 418 mμ ferric maximum we now find half-times of 160 min under aerobic conditions and 50 min under anaerobic conditions (see control values, Table IV).

Table III shows the absorbance and position of the Soret maximum in various mixtures of oxygenated and ferric forms as calculated from the data of Fig. 1. By use of this table relatively accurate half-time values can be obtained even when the spontaneous conversion to the ferric form is incomplete.

Table IV shows the effect of various reducing reagents on the rate of conversion of oxygenated to ferric form. In an evacuated Thunberg cell 8.6 μM cytochrome oxidase was reduced with 100 μM dithionite and the oxygenated compound formed

by rapid aeration of this solution. The cell was then re-evacuated before the addition of the reductant through the side arm. All the reducing reagents listed in Table IV accelerate the rate of conversion of oxygenated into ferric oxidase and with most of these reagents the acceleration is sufficiently high that the spontaneous conversion can be neglected. An additional reagent, tetrachlorohydroquinone, could not be used because it rapidly produced highly colored products on oxidation. The acceleration caused by these reductants, however, is small when compared with the very fast conversion observed in the presence of ferrocytochrome *c*, the half-time of which is measuredin msec (M. V. GILMOUR, unpublished observation). On prolonged action,

TABLE III

ABSORPTION BAND IN MIXTURES OF FERRIC AND OXYGENATED OXIDASE*

Ferric oxidase (%)	λ_{max}	*A*
0	428	1.0
10	428	0.987
20	427	0.979
30	427	0.967
40	426.5	0.956
45	426	0.954
50	425.5	0.944
60	425	0.946
65	424	0.947
70	422	0.948
80	420	0.960
90	419	0.977
100	418	1.0

* Calculated from the values in Fig. 1.

TABLE IV

HALF-TIMES OF THE CONVERSION OF OXYGENATED OXIDASE INTO FERRIC OXIDASE BY REDUCTANT

Cytochrome oxidase concn. 10 μM.

Reductant	*Concn. of H-donor (mM)*	*Half-time (at 425.5 mμ)*	*Final position of Soret band*		*Final ferric oxidase* (%)
			Conditions	*mμ*	
Control (aerobic)	—	160 min	After overnight incubation at 4°	422.2–425	60–70
Control (anaerobic)	—	50 min	After overnight incubation at 4°	420.5	77
Ascorbate	1	7 min	After 32 min	420	80
Guajacol	1.5	7 min	After 45 min	422.7	68
Hydroquinone	1	68 sec	After 60 min (with partial reduction to ferrous)	424	—
Indole acetate	0.1	50 min	Evacuated at 0° overnight	417	100
Indole acetate	0.215	21 min	In 50 min	421.6*	72
Iodide	1.1	Approx. 10 min	In 120 min at room temp.	418	100
Pyrogallol	0.01	45 sec	On further standing partial reduction to ferrous		—
Tetramethyl-*p*-phenylene diamine	0.01	Approx. 45 sec**	Rapidly followed by reduction to ferrous		—

* 0.24 mM KI shifted band to 419.3 (86% Fe^{3+}) in 60 min at room temperature.

** Only approximate because accompanied by some reduction to ferrous.

tetramethyl-*p*-phenylene diamine, and pyrogallol and hydroquinone to a smaller extent, also caused some reduction to the ferrous form. The other reductants, including ferrocytochrome *c*, caused from 70–100 % conversion into the ferric form as indicated by the position of the Soret maximum.

TABLE V

APPARENT BIMOLECULAR RATE CONSTANTS FOR THE CONVERSION OF OXYGENATED INTO FERRIC OXIDASE BY REDUCTANTS

Reductant	*Apparent bimolecular rate constant* ($M^{-1} \cdot sec^{-1}$)
Cytochrome *c*	$7 \cdot 10^6$
Pyrogallol	$2 \cdot 10^3$
Tetramethyl-*p*-phenylene diamine	Approx. $2 \cdot 10^3$
Hydroquinone	10
Ascorbate	2
Indole acetate	2.5
Iodide	>1
Guajacol	1

In order to be able to compare experiments in which different concentrations of reductant were used to achieve a measurable spectral shift, the half-time values (Column 3) were converted into apparent second order rate constants by the formula $k = 2.303/t(a-b) \log b(a-x)/a(b-x)$ or where $a = b$, $k = 1/t \cdot x/a(a-x)$, where a and b are the molar concentrations of reductant and oxidase, respectively, t is the time in seconds and x is the proportion of a and b which have reacted in time t. The value for x was determined by converting the position of the Soret peak at time t into the amount of ferric oxidase formed by the use of Table III. In most instances where $a \gg b$, x can be neglected with regard to a and for $x = 0.5$, $\log b(a-x)/a(b-x)$ becomes $\log 2 = 0.3$. In calculating these rate constants the slow spontaneous conversion has been neglected. These apparent bimolecular rate constants are given in Table V. It must be stressed that we have no direct evidence for the assumption of a true bimolecular reaction. Although all these reductants accelerate the rate of conversion to the ferric enzyme, besides ferrocytochrome *c* which reacts with unique rapidity, only pyrogallol and tetramethyl-*p*-phenylene diamine react rapidly with the oxygenated compound.

Reaction of cytochrome c peroxidase with oxygenated oxidase

Cytochrome *c* peroxidase does not appear to react with oxygenated oxidase. In a differential spectral set-up 10.9 μM cytochrome *c* peroxidase was added to 6.7 μM oxygenated cytochrome oxidase in the sample cuvette and the two reactants in the same concentrations were added to two separate cuvettes for the reference. Scanning from 700 to 350 mμ gave essentially a nil spectrum showing that if the oxygenated compound has a peroxidic function cytochrome *c* peroxidase is not able to react with it. Removal of the oxygenated compound reference cuvette gave a typical absolute spectrum of the oxygenated compound and the addition of hydrogen peroxide (to a final concentration of 440 μM) to the sample cuvette and replacement of the oxygenated compound reference cuvette produced the typical cytochrome *c* peroxidase–H_2O_2 complex difference spectrum. However, approx. 15 min later when

the oxygenated compound reference cuvette was again removed and H_2O_2 added to the peroxidase reference cuvette an altered oxygenated spectrum was obtained. The Soret maximum at 425 mμ was greatly reduced in absorbance and shifted to 430 mμ. This alteration of the oxygenated compound may be due to the destruction of the oxidase enzyme by the high concentration of H_2O_2 or it may indicate some sort of reaction between the cytochrome *c* peroxidase–H_2O_2 complex and the oxygenated compound.

DISCUSSION

The kinetic studies on the dithionite reduction of oxygenated and ferric oxidase have shown a difference between the distinctly biphasic reduction of ferric oxidase and the monophasic reduction of oxygenated oxidase. These findings are at present being analysed at the Johnson Foundation by Dr. M. PRING of Balliol College, Oxford, with the help of analogue computers. The slow component of the reduction of ferric oxidase, ascribed to the reduction of ferricytochrome a_3 is lacking in the reduction of the oxygenated oxidase. Using NADH–phenazine methosulphate as reductant, the difference in the rate of reduction of ferricytochromes *a* and a_3 is not marked, but the oxidation of phenazine methosulphate forms a radical, and this may catalyse, similar to cytochrome *c*, the interaction between ferrocytochrome *a* and ferricytochrome a_3; this is slow in the absence of such mediators. These findings support OKUNUKI's conception of a uniform oxygenated oxidase. This agrees also with the absence of noticeable spectroscopic differences in the compound which can be ascribed to cytochromes *a* and a_3. However, our kinetic findings once again demonstrate the presence of two differently reacting haem-*a* groups, corresponding to cytochromes *a* and a_3 in cytochrome *c* oxidase, originally established by KEILIN AND HARTREE[17]. Attempts to explain the different rates of reduction on the basis of the presence of some oxygenated oxidase in ferric oxidase (OKUNUKI[9], ORII AND OKUNUKI[2,4]) now appear untenable, as the dithionite reductions reported in this paper were carried out with a ferric oxidase preparation with a Soret band at 418 mμ, and any possible formation of oxygenated oxidase during the dithionite reduction was made impossible by removing O_2 before beginning reduction. This is also shown by the complete absence of the stationary phase of reduction which is found in the presence of dissolved O_2.

The phenomenon of the stationary phase of dithionite reduction in the presence of dissolved O_2 after about one-third of the 444 mμ absorbance difference between ferric and ferrous oxidase has been reached, does not necessarily prove that the contribution of ferrocytochrome *a* to that difference is only one-third of the total. With a preparation which contained a molar ratio of 0.58 cytochrome *a* and 0.42 cytochrome a_3, VANNESTE[16] found the contribution of ferrocytochrome *a* to the difference spectrum 33 %, in contrast to the 50 % contribution to the absolute spectrum. This may be due to the greater proximity of the Soret maximum of ferricytochrome *a* (426 mμ) to 444 mμ than that of the ferricytochrome a_3 maximum (414 mμ). However, GILMOUR, WILSON AND LEMBERG[18] while showing the same difference between the Soret maxima of ferricytochromes *a* and a_3 at the temperature of liquid N_2 nevertheless found a cytochrome *a* contribution of about 50 % in the difference spectrum. Some of our observations also support a 50 % contribution of cytochrome a_3. The preparation used by us contained less than 10 % of the non-reducible by-product

(R 425/444 below 0.45)[12,19]. The stationary phase may be a kinetic phenomenon in that the cytochrome a_3-catalysed oxidation of ferrocytochrome *a* by residual O_2 becomes sufficiently fast to balance its further reduction by dithionite before all ferricytochrome *a* had become reduced.

In agreement with WAINIO[20], ferrocyanide in the presence of air forms oxygenated oxidase; this is additional evidence showing that H_2O_2 is not required for its formation (*cf.* refs. 7, 8, 21). The experiments described in this paper show the unexpected persistence of some ferrous oxidase after aeration, a phenomenon already observed by WAINIO (ref. 6, p. 627). This cannot be merely due to continued reduction by non-autoxidizable ferrocyanide, as the same phenomenon has been observed after aeration of ferrous cyanide oxidase ($a^{2+} + a_3{}^{2+}$ CN) and of ferric cyanide oxidase in the presence of dithionite ($a^{2+} + a_3{}^{3+}$ CN + some $a_3{}^{2+}$ CN).

It is well known that cyanide by binding ferric cytochrome a_3 prevents the complete reduction, and the similar phenomenon on dithionite reduction after ferrocyanide may be due to its partial dissociation to give free cyanide. It may well be that cyanide has some effects on the protein as well as on the haem-*a* group (*cf.* CAMERINO AND KING, ref. 22). Our observations confirm the non-autoxidizability of cytochrome *a*.

Our view that oxygenated oxidase is a peroxidic, rather than a reversibly oxygenated compound[7,8] is supported by the findings of the present paper. Similar to the rapid formation of oxygenated oxidase by the action of H_2O_2 on ferrous oxidase, ethyl hydrogen peroxide produces a similar compound. Hydrogen donors able to react with compound III of horse-radish peroxidase[13] are also able to transform oxygenated into ferric oxidase, although none of them approach the effectiveness of cytochrome *c* (see ref. 21). This could be expected in view of the specificity of cytochrome *c* oxidase for its substrate. It must be stressed that oxygenated oxidase while greatly differing from reversibly oxygenated haemoproteins also differs from typical peroxidase–H_2O_2-compounds. In contrast to these, and also to the cytochrome *c* peroxidase–H_2O_2 complex (YONETANI, CHANCE AND KAGIWARA[23]) it is not readily formed from ferric cytochrome oxidase by H_2O_2 or ethyl hydrogen peroxide. This may explain why no reaction between oxygenated oxidase and cytochrome *c* peroxidase has been found. Some compounds formed from ferrous peroxidases with molecular oxygen and H_2O_2 may, however, supply better models for oxygenated oxidase. The acceleration by hydrogen donors of conversion of oxygenated to ferric oxidase indicates that the iron in oxygenated oxidase is not ferric but has higher valency state.

ACKNOWLEDGEMENTS

This work has been carried out during a sabbatical leave's tenure of a visiting professorship of one of us (R.L.) at the University of Pennsylvania, and we are particularly indebted to Professor BRITTON CHANCE for his hospitality which made all the rich resources of his institute available to us with great liberality, and for valuable advice. We also thank Dr. T. YONETANI for his gift of a preparation of crystalline cytochrome *c* peroxidase.

This work has been supported by grants from the National Heart Institute of the National Institutes of Health, U.S.A. No. HE07827, from the Australian

National Health and Medical Research Council and a grant to one of us (M.V.G.) from the Muscular Dystrophy Associations of America.

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