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CYTOCHROME OXIDASE AND ITS DERIVATIVES

IX. SPECTROPHOTOMETRIC STUDIES ON THE RAPID REACTION OF FERROUS CYTOCHROME *c* OXIDASE WITH MOLECULAR OXYGEN UNDER CONDITIONS OF COMPLETE AND PARTIAL OXYGENATION

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SUMMARY

1. The reaction of ferrocytochrome oxidase with molecular oxygen (15 μM) has a half-time of about 3 msec.

2. 100 msec after 2 μM ferrous oxidase is mixed with 15 μM oxygen the difference spectrum of the ferrous enzyme *minus* the mixture shows a minimum in the Soret region at 420 mμ. The difference spectrum of the "oxygenated" compound has a minimum at 421–425 mμ while that of the ferric oxidase lies at 411–412 mμ. Thus under these conditions a mixture of "oxygenated" and ferric forms is produced with the "oxygenated" form predominating.

3. By observing the absorption changes at the wavelength pair 428–418 mμ (in a stop-flow dual wavelength spectrophotometer) it is possible to distinguish between "oxygenated" and ferric oxidase formation in the early stages of the reaction between ferrous oxidase and oxygen. When insufficient oxygen is added so that only 10–30% of the ferrous enzyme reacts with oxygen the product is predominantly ferric oxidase with, on the average, about 30% "oxygenated" compound. The Soret band of this mixture has a broad maximum extending from 426 to 418 mμ. Similar results were obtained with cytochrome *c*-deficient electron transport particle.

4. Vigorous aeration of ferrous oxidase produces "oxygenated" compound only, whereas, mixing with insufficient oxygen produces a mixture of ferric and "oxygenated" forms. It is suggested that "oxygenated" oxidase is formed by the interaction of the primary a_3 FeO_2 complex with ferrocytochrome *a*, whereas ferric cytochrome oxidase (a_3''' + a''') results when the primary complex can interact with ferrocytochrome a_3 as well as with ferrocytochrome *a*.

5. On reduction of the ferric enzyme with dithionite the absorption changes at 428–418 mμ show an initial decreased absorbance followed by the expected increase. The initial absorbance decrease is due to the fast reduction of cytochrome *a* which has a Soret band at 426 mμ in the ferric form. This is followed by the slower reduction of cytochrome a_3 with its Soret band at 414 mμ causing an absorbance increase at 428–418 mμ.

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INTRODUCTION

In three preceding papers[1–3] it has been shown by LEMBERG and co-workers that the end-product of vigorous and complete aeration of ferrous cytochrome *c* oxidase (EC 1.9.3.1) is the "oxygenated" oxidase, first discovered by OKUNUKI[4,5] and later studied by WAINIO and co-workers[6,7]. It differs from ferric oxidase by its absorption spectrum as well as in other properties, *e.g.*, the kinetics of reduction by dithionite[3]. However, the absorption spectra after slow aeration indicated that some ferric oxidase had been produced in addition to "oxygenated" oxidase. The reaction of ferrocytochrome oxidase with molecular oxygen even of low pO_2 is very rapid and is complete within the msec time scale[8]. Previous studies of "oxygenated" cytochrome oxidase have all been carried out at times much greater than this. In view of the possible importance of the "oxygenated" oxidase in the theory of the enzymic mechanism *in vivo* by OKUNUKI[5] and LEMBERG[9] we have investigated whether the initial product of the reaction of ferrous oxidase with molecular oxygen is "oxygenated" or ferric oxidase. In order to distinguish between the two compounds advantage was taken of their spectral differences in the Soret band region. These results are presented in the present paper.

METHODS

Cytochrome c oxidase from beef heart was prepared free from cytochrome *c*, c_1 and *b* as previously described[3] except that the oxidase was finally dissolved in 0.5 % Tween 80. Four preparations of the enzyme (J-1 to J-4) were used. Oxidase concentration is expressed as molarity of haem *a*.

Submitochondrial particles deficient in cytochrome *c* were prepared by washing beef heart mitochondria[11] with KCl solution as described by JACOBS AND SANADI[12] and CHANCE, LEE AND SCHOENER[13], followed by sonication. The preparation designated ('ETP_a') contained about equal parts of electron transport particles and broken mitochondria.

Apparatus (see refs. 14 and 15).

Continuous flow double-beam spectrophotometer. In this apparatus a solution of reduced oxidase from a 200-ml sample chamber first flows past the observation window for the reference light beam, then it is rapidly mixed with air- or O_2-saturated buffer in the ratio 80:1. 100 msec after mixing, the sample flows past the observation window for the measuring light beam. During the time (50 sec) required for the 200-ml sample to flow through the observation tubes the spectrophotometer scans the designated spectral region (80 mμ) and records the difference in absorbance between the sample in the measuring and reference beams. The length of the light path is 1 cm. The spectra given in the figures have been corrected for deviations of the base line from linearity.

Dual wavelength spectrophotometers. Three types were used:

(A) A Beckmann dual monochromator spectrophotometer with a special cuvette holder and attached amplifier and recorder. This instrument has no provision for adding oxygen in the dark, which necessitates switching off the high-voltage amplification during the periods of aeration. Thus, a few seconds delay occurs before the response can be recorded. This instrument was used in the exploratory stage to

test the validity of the method of distinguishing between ferric and "oxygenated" oxidase formation. The light path is 1 cm.

(B) A dual wavelength spectrophotometer with a recording oscilloscope and a 30-ml volume stop-flow attachment which is manually operated (B_1). A similar instrument with a volume capacity of 7 ml was also used (B_2). The reduced enzyme is mixed with air- or O_2-saturated buffer in the ratio 100:1. The length of the light path is 0.1 cm.

(C) A dual wavelength spectrophotometer with a recording oscilloscope and an 80-ml volume, pneumatically driven stop-flow attachment. This mixes reduced enzyme with air- or O_2-saturated buffer in a ratio of 80:1. It has a lightpath of 1 cm. In some experiments this instrument was used without stop-flow attachment.

Distinction by dual wavelength spectrophotometry between ferric and "oxygenated" oxidase formation from ferrous oxidase. In Fig. 1 (taken from ref. 3, Fig. 1) the region between the respective maxima (428 and 418 mμ) of "oxygenated" and ferric oxidase is indicated by solid lines. In this region the decrease in absorption of the ferrous enzyme towards shorter wavelengths is almost parallel to the decrease in absorption

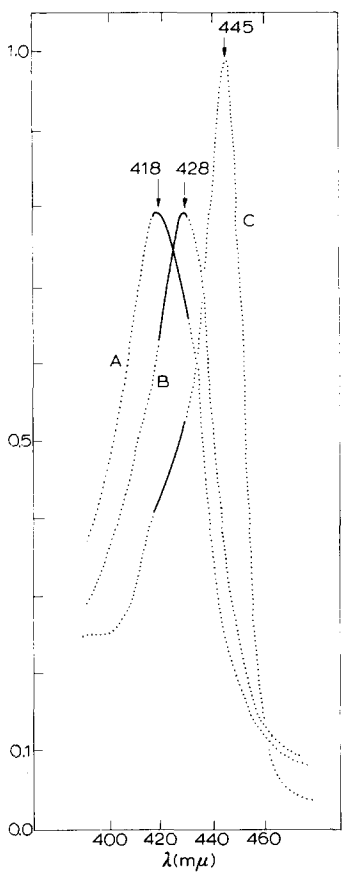


Fig. 1. Absorption curves of ferric (A), "oxygenated" (B) and ferrous (C) oxidase in the Soret band region according to LEMBERG AND GILMOUR[3]. The parts chosen for differential dual wavelength spectrophotometry are in solid lines. Oxidase 8.4 μM.

of "oxygenated" oxidase in the same region, so that on conversion of one to the other there would be no or only a slight change observed for the absorption difference 428–418 mμ. The absorption of the ferric enzyme, however, increases from 428 to 418 mμ, and its formation from ferrous enzyme would therefore be accompanied by a substantial decrease of the absorption difference 428–418 mμ, as shown by the following sample calculation for which the data from Fig. 1 were used. At the wavelength pair 428–418 mμ the absorbance of ferrous oxidase is 0.51 — 0.42 = 0.09, that of ferric oxidase is 0.69 — 0.79 = —0.10; the change on conversion from ferrous to ferric oxidase is thus —0.19. For the "oxygenated" compound this absorbance is 0.15 and the change on conversion from ferrous to "oxygenated" oxidase is almost negligible. Table I shows that the wavelength pair for a nil effect for formation of the "oxygenated" enzyme depends somewhat upon the enzyme preparation, the gas used to oxygenate, and the concentration of dithionite used to reduce the enzyme before oxygenating. There is no wavelength pair which gives a nil effect for the formation of ferric oxidase with a substantial change for the formation of "oxygenated" oxidase. For most experiments we have used the wavelength pair 428–418 mμ and assumed a zero value for the formation of "oxygenated" oxidase.

TABLE I

OPTIMAL WAVELENGTH PAIRS FOR DIFFERENTIATION OF TRANSFORMATION OF FERROUS INTO "OXYGENATED" OR FERRIC OXIDASE

Oxidase preparation	*Dithionite concn. (μM)*	*Gas*	λ_{max}	$\Delta\lambda_{max}$*	*Nil value for "oxygenated" oxidase*
1	100	O_2	428	430.5–417	431–418
1	100	O_2	428	431–417	429.5–418
2	80	O_2	427	429–417	429–418
1	4570	O_2	426.5	431–417	427–418
2	250	Air	426.7	429–417	429–418
1	540	Air	425	429–417	427–418
1	133	N_2 with 0.5 % O_2	426	427.5–418	428–417

* In some instances 412 mμ would also be a suitable reference wavelength.

Where insufficient oxygen was added so that only a portion of the ferrous oxidase reacted, the amount of reacting enzyme was computed by using the wavelength pair 444–462 mμ. 444 mμ is the maximum of the ferrous enzyme and 462 mμ is a convenient isosbestic point. The ratio $\Delta A_{428-418\ m\mu}/\Delta A_{444-462\ m\mu}$ was then used to determine ferric or "oxygenated" oxidase formation. When ferric oxidase is formed from ferrous the value of this ratio is 0.27, whereas when "oxygenated" oxidase is formed this value is assumed to be zero.

RESULTS

Dithionite reduction of ferric and "oxygenated" oxidase

The kinetics of the reduction of cytochrome oxidase by dithionite found by LEMBERG AND GILMOUR[3] with the Cary recording spectrophotometer are confirmed with the dual wavelength instrument where the reduction is observed at the wave-

length pair 444–462 mμ. The reaction for the ferric enzyme, shown in Fig. 2, is distinctly biphasic.

Reduction of the "oxygenated" compound of cytochrome oxidase is rapid and monophasic. This is seen in Fig. 4 (increase of absorption following oxygenation).

Rate of reaction of ferrous oxidase with oxygen

Tracing of the absorbance change at the wavelength pair 444–462 mμ *versus* time for the reaction of ferrous oxidase with oxygen is shown in Fig. 3. The reaction trace shows a decreased absorbance before the end of the plunger movement indicating that the reaction is more than 50 % complete during the mixing time. The pseudo first-order velocity constant is computed from the 4 msec time after mixing and the spectrophotometric deflections to be 220 sec^{-1} corresponding to a half-time of 3.1 msec. The second-order velocity constant is computed to be $1.5 \cdot 10^7\ M^{-1} \cdot sec^{-1}$. In similar experiments, GIBSON AND GREENWOOD[8] obtained a value of $3 \cdot 10^7$–$6 \cdot 10^7\ M^{-1} \cdot sec^{-1}$ for the second order with low oxygen concentration (less than 5 μM) and an equivalent amount of oxidase. SCHINDLER[16] reports a similar value. Cytochrome *c* is not required for the reaction.

Ferrous oxidase reacts far more rapidly with oxygen than does dithionite which has a first-order velocity constant of 42 sec^{-1} (ref. 17). This was measured with $8 \cdot 10^{-5}$–$48 \cdot 10^{-5}$ M dithionite and did not depend on oxygen concentration from

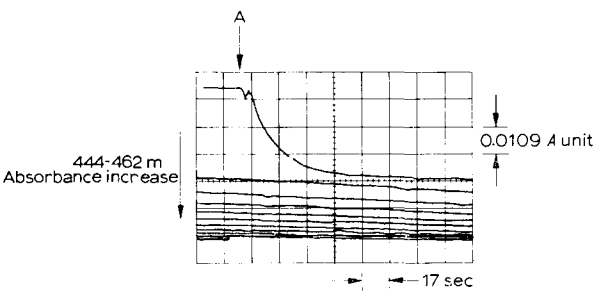


Fig. 2. Dithionite reduction of ferric oxidase ($a a_3$). Dual wavelength spectrophotometer (C) with 1-cm open cuvette $\lambda_{444-462\ m\mu}$. To 0.86 μM ferric oxidase J-2 saturated with N_2, dithionite was added at A to a concentration of 500 μM.

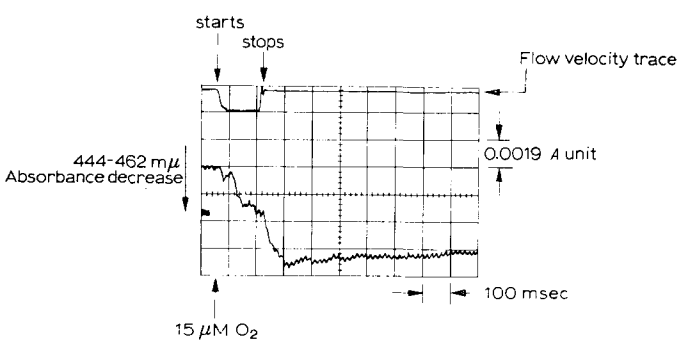


Fig. 3. Oxygenation of ferrous oxidase. Dual wavelength spectrophotometer (C) with stopped flow. $\lambda_{444-462\ m\mu}$. Oxidase J-1 1.07 μM, dithionite 0.3 mM. Oxygen to 15 μM with oxygenated buffer. The ferric oxidase solution was saturated with N_2 before dithionite was added and 30 min allowed for reduction. The top trace records the plunger movement.

$10 \cdot 10^{-5}$–$48 \cdot 10^{-5}$ M. In studying the oxidase–oxygen reaction the dithionite effect can therefore be ignored except in the presence of high concentrations of dithionite where the reaction becomes complex as shown in Fig. 4. During these additions of oxygen, while absorbance changes were observed, a vibrating oxygen electrode, inserted into the solution underneath a layer of liquid paraffin, did not detect the presence of

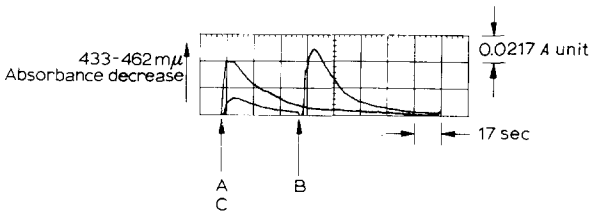


Fig. 4. Vigorous oxygenation of ferrous oxidase in the presence of high dithionite concentration. Dual wavelength spectrophotometer (C) with open 1-cm cuvette under a layer of liquid paraffin. $\lambda_{443-462\ m\mu}$. Oxidase J-1 1.05 μM, reduced 30 min by 5 mg dithionite (final concn. about 8 mM). At A, and C successively aerated with 3-sec bursts of O_2.

oxygen (oxygen electrode trace not shown) which emphasizes the rapidity with which oxygen is reacting in this system. The initial rapid absorbance decrease at 443–462 μM can be attributed to the reaction of ferrous oxidase with oxygen, the subsequent slowing down phase represents a composite of this reaction and the reaction of dithionite with the oxidation product(s) of ferrous oxidase. Finally, when all the oxygen has reacted, only the latter reaction occurs returning the absorbance to that of the original fully-reduced level. The monophasic character of this reduction curve indicates that the oxidation product which was formed from ferrous oxidase *plus* oxygen in this experiment is predominately "oxygenated" oxidase. The observed maximal absorbance change in Curve B corresponds to 70 % of the theoretical value for the formation of "oxygenated" form and if this curve is extrapolated to zero reduction time, this would be 90 %.

Difference spectrum of ferrous oxidase minus ferrous oxidase plus oxygen 100 msec after mixing

The difference spectra calculated from Fig. 1 for ferrous *minus* ferric and ferrous *minus* "oxygenated" oxidase are given in Fig. 5 (Curves A and B, respectively). Its ferrous *minus* ferric minimum was at 412 mμ, its ferrous *minus* "oxygenated" minimum was at 421 mμ.

The "oxygenated" compound was unusually labile; this was probably due to the rather high temperature (about 30°) to which the enzyme solution was exposed in the cell compartment of the spectrophotometer. In 30 min the 421 minimum had moved to 414 mμ; this may explain the difference from its calculated position at 425 mμ.

The Soret minimum representing the oxidation product of the reaction was found at 420 mμ in two experiments, one with oxygen-saturated, one with air-saturated buffer. The minimum is thus closer to that expected for "oxygenated" than for ferric oxidase. Assuming a linear shift of the minimum in mixtures of "oxygenated" and ferric oxidase, the ferric oxidase content of the product for the reaction observed can be computed to be 10–30 %.

In experiments in which reduction was carried out by ascorbate and tetramethyl-*p*-phenylenediamine in the presence of small amounts of cytochrome *c* (one fourth to one half of the amount equivalent to the heme *a* of the oxidase), the minimum of the end-product was 418 mμ. The spectrum was scanned 50 msec after mixing (unpublished experiments of Dr. M. GILMOUR).

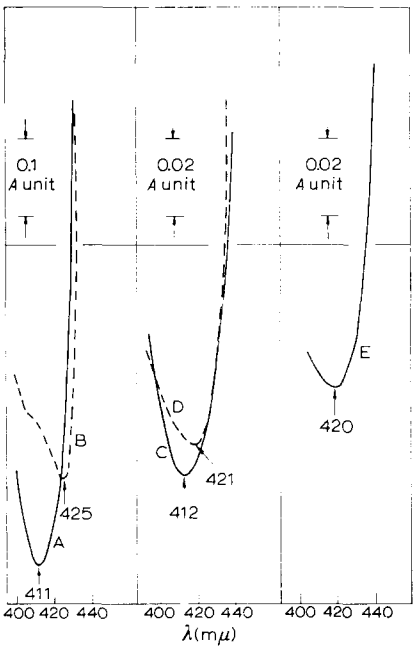


Fig. 5. Ferrous *minus* oxygenated oxidase spectra 100 msec after oxygenation. Curve A, ferrous *minus* ferric oxidase spectrum. Curve B, ferrous *minus* "oxygenated" difference spectrum calculated from Fig. 1. Curve C, ferrous *minus* ferric oxidase spectrum. Curve D, ferrous *minus* "oxygenated" oxidase spectrum recorded in the continuous-flow instrument. Oxidase J-4 8.4 μM. Oxygenation as for Fig. 1. Curve E, ferrous *minus* ferrous oxidase *plus* oxygen 100 msec after mixing in the continuous-flow attachment. A 2 μM ferric oxidase solution was saturated with N_2 and reduced 30 min in the larger chamber of the instrument, then mixed with oxygen-saturated phosphate buffer in the ratio 80:1; final oxygen concentration 15 μM. 1-cm cuvette.

Distinction between "oxygenated" and ferric oxidase by $A_{428-418\ m\mu}$

Exploratory studies with the Beckmann dual wavelength spectrophotometer

These studies done on a comparatively long time-scale test the validity of using the 428–418 mμ wavelength pair to distinguish between "oxygenated" and ferric oxidase. The instrument used has been described under METHODS (Section A).

These experiments (*cf.* also Fig. 5 of ref. 30) show the applicability of the method and confirm that the product of vigorous aeration by short bursts of air of a few seconds duration is "oxygenated", not ferric oxidase. This is slowly transformed into ferric oxidase on standing for about 2 h.

Rapid stop-flow studies observed at 428–418 mμ

Dual wavelength spectrophotometers with stop-flow attachment were used for the study of rapid partial oxygenation. The results are collected in Table II. The reactions were observed approx. 4 msec after mixing in the pneumatically driven

TABLE II

PRODUCT OF RAPID PARTIAL OXYGENATION

Spectrophotometer	*Oxidase* (μM)	*Dithionite* (μM)	*Oxygenation by buffer*	O_2 (μM)	*Oxygenation* (%)	$\frac{\Delta A_{428-418\ m\mu}}{\Delta A_{444-462\ m\mu}}$ *	*Ferric oxidase* (%)
B_2	1.85	3000	Aerated 1 → 100	3	8	0.16	60
B_2	1.85	3000	Aerated 1 → 100	3	10–11	0.18	66
C	1.07	300	Oxygenated 1 → 80	15	10.5	0.21	83
B_1	8.0	800	Oxygenated 1 → 100	13	11	0.165**	59
B_1	8.0	5000	Oxygenated 1 → 100	13	13	0.218***	84
B_1	8.0	5000	Aerated 1 → 100	2.6	6.5	0.20	74
B_1	8.0	1000	Oxygenated 1 → 100	13	33	0.228	80
Average						0.196	70 (56–84)

See for spectrophotometers, METHODS. The ferric oxidase solution was saturated with N_2 and reduced for 30 min by dithionite in the large syringe of the apparatus. The small syringe contained air- or oxygen-saturated water.

* 0.27 for ferric oxidase; 0 for "oxygenated" oxidase formation.

** Six measurements at 444–462 mμ, two at 428–418 mμ.

*** Four measurements at 444–462 mμ, four at 428–418 mμ.

stop-flow apparatus, with the oscilloscope sweep speed set at 100 msec or 17 sec/cm. Each reading at 428–418 mμ was accompanied by a reading at 444–462 mμ in order to determine the amount of enzyme reacting. In these experiments oxygenation was only 6–33 % complete. The results are expressed as the ratio $\Delta A_{428-418\ m\mu}/\Delta A_{444-462\ m\mu}$ from which the ferric and "oxygenated" oxidase content of the reaction product can be determined. As described under METHODS, this ratio is 0.27 for ferric oxidase formation, but close to zero for "oxygenated" oxidase formation. The ratio varied from 0.15 to 0.23, indicating that the final product consisted of 56–82 % ferric *plus* 44–18 % "oxygenated" oxidase.

The spectrum of this mixture showed a broad Soret band extending from 418 mμ (or below) to 426 mμ. This spectrum was obtained using the same experimental set-up as above but recording the absorbance change on oxygenation at 2-mμ wavelength intervals from 430 to 418 mμ all *minus* the 462-mμ isosbestic point. The results are presented in Table III. A comparison of this spectrum (Column 4) with that (Column 5) of ferric oxidase in which $A_{418-462\ m\mu}$ has been arbitrarily put to 1, shows that there appear to be two maxima, a larger one at 418 mμ (perhaps $<$ 418 mμ) and a second weaker one at 428–426 mμ. The flat maximum is therefore probably composed of two maxima, those of the ferric and of the "oxygenated" enzyme.

A comparison of the responses at 605–632 mμ with those at 444–462 mμ showed that the $\Delta\gamma/\alpha$ ratio (*cf.* ref. 19) was 5.90 $\pm$ 0.18 in four experiments; that of the ferrous *minus* ferric oxidase was 5.78, that of the ferrous *minus* "oxygenated" oxidase 6.62. These results again indicate the product to be a mixture.

Experiments with cytochrome c-deficient submitochondrial particles

The preparation of these particles has been described above. In the experiments the ratio $\Delta A_{428-418\ m\mu}/\Delta A_{444-462\ m\mu}$ was not measured directly, but was obtained by subtraction from $\Delta A_{428-462\ m\mu}/\Delta A_{444-462\ m\mu}$ and $\Delta A_{418-462\ m\mu}/\Delta A_{444-462\ m\mu}$. These

TABLE III

SORET BAND MAXIMUM OF THE PRODUCT OF RAPID PARTIAL OXYGENATION

$\Delta\lambda$ ($m\mu$)	*Number of measurements*	$\Delta A_{av.}$	$\frac{\Delta A}{\Delta A_{418-462\ m\mu}}$ *found*	$\frac{\Delta A}{\Delta A_{418-462\ m\mu}}$ *for ferric oxidase*	*Difference between product and ferric oxidase*
418–462	2	0.0057	1	1	0
420–462	3	0.0046	0.807	0.947	−0.14
422–462	1	0.0047	0.825	0.85	−0.025
424–462	1	0.0046	0.807	0.752	0.055
426–462	1	0.0046	0.807	0.645	0.162
428–462	5	0.0039	0.65	0.475	0.175
430–462	1	0.0021	0.368	0.360	0.008
445–462	5	0.0088	—	—	—

parameters as well as the ratio $\Delta A_{428-462\ m\mu}/\Delta A_{418-462\ m\mu}$ are given in Table IV. In most experiments oxygenation was more complete by replacing oxygenation with aerated buffer by oxygenation with catalase (hydrogen-peroxide:hydrogen-peroxide oxidoreductase, EC 1.11.1.6). To approx. 1 mM H_2O_2 in the small syringe 0.5 μM catalase was added immediately before addition to the ferrous oxidase. Thus in Expt. 131 the degree of oxygenation achieved was 2.6 times that achieved by aerated buffer (Expt. 127). The percentages of ferric oxidase given in the table can only be considered as rough estimates. Most of the parameters, however, lie between those calculated for the formation of ferric and the formation of "oxygenated" oxidase. Expt. 134 shows no difference between $\Delta A_{424-462\ m\mu}$ and $\Delta A_{418-462\ m\mu}$ indicating again a broad Soret maximum from 418 to 424 mμ.

If 10 mM succinate was used as reductant (Expt. 132) the product contained a greater percentage of ferric oxidase than when the reductant was dithionite. In Expt. 127 carried out with a less pure preparation ('ETP_a') and addition of air-saturated buffer instead of H_2O_2 *plus* catalase, only ferric oxidase was found while in a similar experiment with pure electron transport particles and H_2O_2 *plus* catalase (Expt. 132) some "oxygenated" oxidase was present.

Observations on the reduction of ferric oxidase at 428–418 mμ

Reduction of ferric oxidase by dithionite at 428–418 mμ initially causes a rapid decrease of absorbance which is followed by a larger slow increase (Fig. 6).

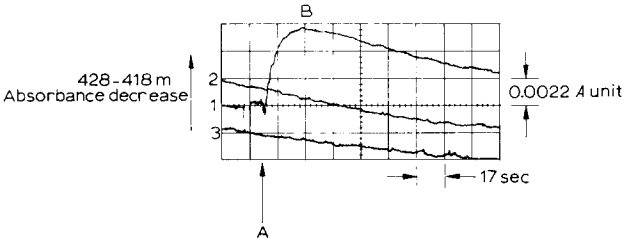


Fig. 6. Reduction of ferric oxidase (aa_3) at 428–418 mμ. Dual wavelength spectrophotometer with 1-cm open cuvettes under liquid paraffin, 0.86 μM oxidase J-2. 1–3 successive traces. A, addition of dithionite to 0.5 mM concentration. Absorbance decrease (upward deflection) is 2.8 divisions. B, beginning of absorbance increase, not fully shown; total increase 7.3 divisions; a similar change of 7.5 divisions is calculated from the data in Fig. 1 ($0.27 \times \Delta A_{444-462\ m\mu}$).

TABLE IV

OXYGENATION OF CYTOCHROME *c*-DEFICIENT SUBMITOCHONDRIAL PARTICLES

For electron transport particles (ETP) and less pure ETP (ETP_a) see under METHODS. Final concn. 0.5–1.0 mg protein per ml.

Expt. No.	*Particle*	*Reductant*	*Reduction time (min)*	*Oxygenation*	$\frac{\Delta A_{428-462\,m\mu}}{\Delta A_{444-462\,m\mu}}$	$\frac{\Delta A_{418-462\,m\mu}}{\Delta A_{444-462\,m\mu}}$	$\frac{\Delta A_{428-462\,m\mu}}{\Delta A_{418-462\,m\mu}}$	$\frac{\Delta A_{428-418\,m\mu}}{\Delta A_{444-462\,m\mu}}$*	*Ferric oxidase (approx. %)*
131	ETP_a	Dithionite (3 mM)	10	Catalase–H_2O_2	0.46	0.56	1.24	0.10	40
133	ETP	Dithionite (3 mM)	10	Catalase–H_2O_2	0.49	0.47	0.94	−0.02	0
134	ETP	Dithionite (3 mM)	10	Catalase–H_2O_2	0.42	0.49**	1.17	0.07	27
132	ETP	Succinate (10 mM)	15	Catalase–H_2O_2	0.30	0.53	1.78	0.23	85
127	ETP_a	Succinate (10 mM)	15	Aerated buffer	0.22	0.58	2.65	0.36	100
				Ferric oxidase	0.27	0.54	1.98	0.27	100
				Oxygenated oxidase	0.45	0.32	0.74	0	0

* Calculated from $\Delta A_{428-462\,m\mu}/\Delta A_{444-462\,m\mu} - \Delta A_{418-462\,m\mu}/\Delta A_{444-462\,m\mu}$.

** $\Delta A_{424-462\,m\mu}/\Delta A_{444-462\,m\mu} = 0.48$.

This phenomenon is not observed at 444–462 mμ or with some other wavelength pairs. The initial absorbance decrease is somewhat variable. Fig. 7 shows that the absorbance at both 428 and 418 mμ immediately decreases on reduction but this decrease is initially faster at 428 mμ than at 418 mμ; then it slows down to a rate below that of the 418 mμ decrease. The maximal difference is reached after 30 sec and both have become equal after 130 sec. Finally, the decrease at 418 mμ exceeds that at 428 mμ so that the $\Delta A_{428-418\ m\mu}$ now gives an increase in absorbance. In another experiment the spectrum of the Soret region was scanned at intervals during the first 10 sec after dithionite addition and the initial absorbance decrease was also observed.

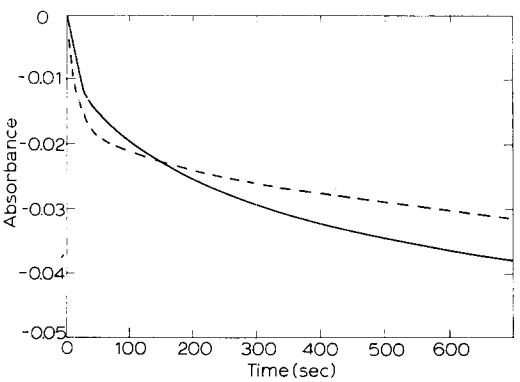


Fig. 7. Reduction of ferric oxidase. ———, 418–462 mμ; – – – –, 428–462 mμ. Dual wavelength spectrophotometer with 1-cm cuvettes. 0.86 μM oxidase J-2 reduced in both experiments by 0.5 mM dithionite.

This phenomenon can also be deduced from Fig. 16 (Curves 1 and 2) of LEMBERG AND MANSLEY[18]. The results can be interpreted as due to differences between ferricytochrome *a* and a_3 in their rate of reduction by dithionite and in their ferric spectrum with the initial reduction of ferricytochrome *a* causing the initial absorbance decrease[19,23]. Its Soret absorption maximum for the difference spectrum is at 426 mμ whereas that for ferricytochrome a_3 is 412 mμ (ref. 20). This is for spectra recorded at low temperature, but difference spectra calculated from room temperature absolute spectra gave similar results[21]. The presence of "oxygenated" oxidase formed by residual oxygen in the solution cannot explain the observed phenomenon for as has been shown above, $\Delta A_{428-418\ m\mu}$ is practically nil for its formation from ferrous oxidase. Also the 418 mμ Soret maximum for our ferric enzyme preparation indicates that it does not contain a significant amount of "oxygenated" compound.

DISCUSSION

Previous experiments of LEMBERG and co-workers[1–3] have shown that the product of the reaction of ferrous cytochrome oxidase with an excess of molecular oxygen is the "oxygenated" oxidase of OKUNUKI, but that under conditions of slow or incomplete oxygenation the product contained some ferric oxidase, indicated by a Soret maximum at shorter wavelengths than 428 mμ. Recently, WITTENBERG *et al.*[28] have shown that rapid and complete oxygenation of horse radish ferroperoxidase

yields an "oxyperoxidase" which in many of its properties resembles "oxygenated" oxidase, whereas slow oxygenation yielded ferric peroxidase.

In the present paper, the product of the very rapid (msec) but incomplete (5–30 %) oxygenation formed from ferrous cytochrome *c* oxidase with small concentrations (3–15 μM) of oxygen in the presence of 0.1–5 mM dithionite is investigated. It is found to be a mixture of ferric and "oxygenated" oxidase, containing varying amounts (10–80 %) of the ferric enzyme. It appears that the less complete the oxygenation, the more ferric rather than "oxygenated" oxidase is formed.

These findings show that neither of the two main theories of the reaction mechanism of cytochrome *c* oxidase is sufficient to explain all the facts. In the classical theory, based on Keilin's original distinction of cytochromes a_3 and *a*, ferrocytochrome *c* is oxidized by ferricytochrome *a*, and ferrocytochrome *a* in turn by ferricytochrome a_3 which is formed by autoxidation of ferrocytochrome a_3 in a non-specified manner, the scheme being: electron donors $\rightarrow c \rightarrow a \rightarrow a_3 \rightarrow O_2$. This theory does not take account of the formation of "oxygenated" oxidase by autoxidation of the ferrous enzyme. Although it is readily formed from ferrous (but not from ferric oxidase) with H_2O_2, it is also formed by the action of molecular oxygen on ferrous oxidase under conditions under which the reductant does not form H_2O_2 with oxygen, *e.g.* with formamidinosulphinic acid[2,29,30], ferrocytochrome *c*[2,31] and potassium ferrocyanide[3]. The classical theory has also no satisfactory explanation for the fact that the reduction of ferricytochrome a_3 by ferrocytochrome *a* is a slow reaction in the purified, cytochrome *c*-free, oxidase preparation (*cf.* refs. 18, 19).

The experiments presented in this paper show that our preparation, like that of GIBSON AND GREENWOOD[8], is able to react rapidly with molecular oxygen in the absence of cytochrome *c*. Purified oxidase does not appear to differ from the enzyme present in the cell by a decreased activity of its copper, a possibility discussed by GIBSON[25]. The work of GILMOUR[22] has shown that the copper is highly reactive.

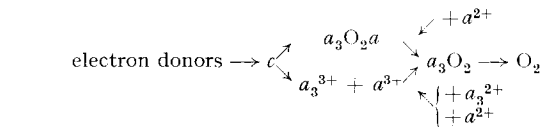
The theory of OKUNUKI[5] postulates that cytochrome *c* is an integral part of the oxidase, not only as electron donor, but as a constituent which has an allosteric effect on cytochrome "*a*", no distinction being made in this theory between cytochrome *a* and a_3. In this theory, "oxygenated" oxidase is assumed to be the compound rapidly reacting with cytochrome *c* in the ternary complex. His scheme is (ref. 5, p. 243):

$$\text{electron donors} \longrightarrow c_1 \longrightarrow \begin{bmatrix} a \cdot O_2 \\ \Uparrow\Downarrow \\ c \end{bmatrix}$$

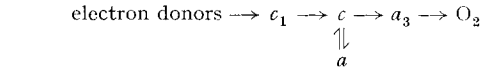
The present experiments cannot be explained on this basis alone. The interaction of ferrous oxidase with molecular oxygen does not require cytochrome *c*. "Oxygenated" oxidase, once formed, is comparatively stable in the absence of cytochrome *c*. Yet in the rapid (msec) and incomplete oxygenation in the presence of excess dithionite ferric, not "oxygenated" oxidase is the main product of the reaction with oxygen. Without postulating cytochromes *a* and a_3, this could only be explained by assuming a very rapid interaction between ferrous and "oxygenated" oxidase, to form ferric oxidase. However, we have occasionally found ferrous and "oxygenated" oxidase to coexist for periods of time considerably longer than the milliseconds of the formation of ferric oxidase in the partial rapid oxygenation described in this paper (*cf.* ref. 1, *e.g.* Fig. 4 and ref. 2, p. 5).

The following hypothesis appears to be able to explain these results. It is postulated that the original Fe^{2+}–O_2 complex of cytochrome a_3, too reactive to be observable spectrophotometrically, can interact with ferrocytochrome a_3 as well as with ferrocytochrome a. On vigorous aeration, ferrocytochrome a_3 disappears rapidly and only ferrocytochrome a is left to react with the oxygen complex of cytochrome a_3, forming "oxygenated" oxidase, whereas in partial oxygenation ferrocytochromes a_3 and a can both interact with the oxygenated complex of cytochrome a_3. In the following scheme "oxygenated" oxidase is abbreviated a_3O_2a indicating that both cytochromes a and a_3 have participated in its formation, without postulating a specific structure for the compound. It may, *e.g.* consist of mixtures of a_3^{4+} with a^{3+} *or* a^{4+}.

Schemically this can be represented as:



This scheme (*cf.* ref. 31) is nearer to that of YAKUSHIJI AND OKUNUKI[26]



than to the more recent scheme of OKUNUKI shown above, although it does not include the "oxygenated" oxidase. There are some possible relations of our present scheme to that of NICHOLLS (ref. 27, Scheme 1) in so far as it also postulates two sites for the interaction of oxidase with cytochrome *c*.

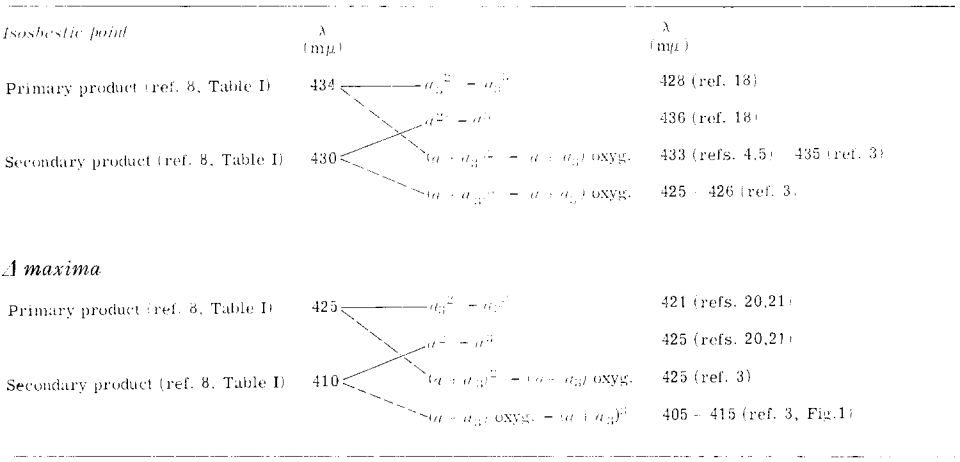
In two recent papers published after the completion of our experimental studies, GREENWOOD AND GIBSON[10] and WHARTON AND GIBSON[29] have investigated the extremely rapid oxygenation of ferrous oxidase liberated by flash photolysis from its CO-compound. The results of these two papers somewhat differ. In the first, the initial reaction of oxygenation (Δ maximum 425 mμ, isosbestic point 434 mμ) is interpreted as formation of a_3^{3+} from a_3^{2+} the later reaction as formation of a^{3+} from a^{2+} while a still later increase at 434 mμ is ascribed to the formation of oxygenated oxidase. Table V shows that these observations of GREENWOOD AND GIBSON[10] may be better interpreted by the assumption that the primary product is "oxygenated" oxidase, followed by its conversion to ferric oxidase ($a_3^{3+} + a^{3+}$). The authors assume that the "dynamic" isosbestic points would differ from the "static" ones, but there is no real reason why this should be so. The isosbestic points and Δ maxima listed in Table V for cytochromes a and a_3 are based on many experiments of ourselves and others[21] and take account of autoxidizibility, reactivity with CO and cyanide, γ/α ratios and rates of reduction by dithionite. It has, for example, been confirmed by GIBSON and co-workers[23,24] that ferricytochrome a is reduced more rapidly than ferricytochrome a_3 by dithionite. In fact, these "static" spectra are also difference spectra which would not be affected *e.g.* by the presence of non-reducible altered oxidase, nor does cytochrome a react with CO.

In the later paper, WHARTON AND GIBSON[29] find after 1.5 msec of oxygenation a flat Δ maximum at 416 mμ and the isosbestic point at 432 mμ, alterations closer to those expected for the formation of ferric oxidase from ferrous, and in substantial

TABLE V

PROPERTIES OF THE PRIMARY AND SECONDARY OXYGENATION PRODUCT OF FERROUS CYTOCHROME OXIDASE ACCORDING TO GREENWOOD AND GIBSON[10]

———, identification suggested by GREENWOOD AND GIBSON; – – – –, more likely identification suggested by authors of this paper.



agreement with our experimental results on very incomplete oxygenation reported in this paper. Later, in a few secs "oxygenated" oxidase is formed. They conclude from these experiments that ferric oxidase is the precursor of "oxygenated" oxidase. However, ferric oxidase is known to be quite stable and cannot be transformed into "oxygenated" without preceding reduction. The authors indeed mention that reductants may play a role in the formation of "oxygenated" oxidase. Thus ferric oxidase can hardly be considered as precursor of oxygenated, and the reaction is more likely to be ferrous $\nearrow$ "oxygenated" / $\searrow$ ferric than ferrous → ferric → "oxygenated". Further experiments are required to establish the nature of "oxygenated" oxidase and its role in the enzymic mechanism. There may be another form of oxidized oxidase different from stable ferric oxidase and spectroscopically differing from it by the flat Soret band. However, the results of our paper rather support the assumption that the flatness of this band, in contrast to the sharper band of ferric oxidase, is due to admixture of "oxygenated" to ferric oxidase.

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