BBA 45559

CYTOCHROME OXIDASE AND ITS DERIVATIVES

VII. FURTHER OBSERVATIONS ON THE COMPOUND FORMED BY THE REACTION OF FERROUS CYTOCHROME *c* OXIDASE WITH OXYGEN AND HYDROGEN PEROXIDE AND ON ITS REACTIONS

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(Received December 22nd, 1966)

SUMMARY

Molecular O_2 and H_2O_2 acting upon ferrous cytochrome *c* oxidase (EC 1.9.3.1) appear to form the same compound. Under conditions of complete formation by O_2 or by H_2O_2, the Soret band lies at 427–428 mμ and is of about equal height to the Soret band of the ferric oxidase at 418 mμ. This makes the interpretation of its absorption curve as one of a mixture of ferrous and ferric oxidase untenable. While formation of the 428-mμ compound is complete when 3 moles of H_2O_2 are added to 1 haem *a* equivalent of oxidase reduced by 4 moles of dithionite a greater excess of molecular O_2 (8–20 moles per mole of haem *a*) must be added to the gas phase. The 428-mμ compound can, however, be formed under conditions under which no H_2O_2 is formed by the autoxidation of the reductant, *e.g.* formamidinosulphinic acid or 1 mole of ferrocytochrome *c*.

The effects of various conditions (standing in air and after evacuation) and reagents (CO, ferricyanide and catalase) are studied. They show that the compound cannot be a reversibly oxygenated compound such as oxyhaemoglobin.

INTRODUCTION

In a preceding paper[1] the reaction of molecular O_2 and of H_2O_2 with cytochrome *c* oxidase reduced by dithionite has been reported and the work of other authors on this subject has been quoted. In contrast to ORII AND OKUNUKI[2,3] no significant differences were found between the compound formed with either reagent. The observations reported in the present paper confirm that they are spectroscopically indistinguishable. If either reaction is allowed to go to completion, the Soret band of the resulting compound lies at 427–428 mμ and is of almost equal height to that of the ferric enzyme at 418 mμ. This confirms the earlier findings of OKUNUKI *et al.*[4], of WAINIO[5], DAVISON AND WAINIO[6], and of LEMBERG AND MANSLEY[1] that a new form of the oxidase is formed in these reactions and excludes the possibility[7,8] that the spectrum may be due to a mixture of ferric and ferrous enzyme.

Although it is shown in the present paper that a smaller excess of H_2O_2 than of molecular O_2 is required for complete formation of the 428-mμ compound, other evidence already discussed in the paper of LEMBERG AND MANSLEY[1] and set out more fully in the present paper, shows that ferrous oxidase can react directly with molecular O_2 without the preceding formation of H_2O_2 by autoxidation of the reductant.

The findings in this paper on the effect of various reagents on the rate of conversion of the 428-mμ compound to the ferric enzyme deviate only in detail from those reported previously; again the effects were the same whether the compound had been formed by molecular O_2 or H_2O_2. It is also confirmed that the 428-mμ compound does not lose O_2 on evacuation to give the ferrous compound, nor is O_2 replaced by CO to give a mixture of CO–cytochrome a_3 with ferric or ferrous cytochrome *a*.

MATERIALS AND METHODS

Cytochrome oxidase

Purified enzyme was prepared as described by MANSLEY, STANBURY AND LEMBERG[9] and was stored deep-frozen in small amounts. The peak of the Soret (γ) band of the fresh ferric enzyme was at 418–419 mμ in all preparations. If a thawed sample was maintained at 4–5° for a number of weeks, the peak moved to longer wavelengths, *e.g.* a preparation with the original band position at 418.2 mμ moved to 423 mμ in 6 weeks. The Soret band of the ferrous enzyme, however, remained at 443–444 mμ. The purity of a preparation was gauged from the ferrous spectrum. A ratio $A_{605m\mu}/A_{554m\mu}$ of 3.0 indicates absence of cytochromes c_1 and *b*, a ratio $A_{424m\mu}/A_{443m\mu}$ of 0.45 indicates virtual absence of non-reducible enzyme (*cf.* refs. 10,11). In the preparations used in this paper, the first ratio was 2.0–3.0, in most instances 2.7–3.0, while the second ratio was usually 0.45–0.55.

Reagents

Catalase, prepared from bovine liver was kindly supplied by Dr. D. B. MORELL of this laboratory. CO was generated in a glass apparatus by dropping formic acid into concentrated H_2SO_4 and was freed from O_2 by passage through an alkaline pyrogallol trap. Formamidinosulphinic acid was obtained from Aldrich Chemical Co., Wisc., U.S.A. Dry sodium dithionite was diluted by solid sucrose (A. R. Townson and Mercer, Sydney) as previously described[12].

Reduction of the enzyme

The reduction of cytochrome oxidase to the ferrous state was carried out in a specially constructed Thunberg tube which fitted into the sample compartment of the spectrophotometer. The ferric enzyme (approx. 0.05 μmole haem *a* in 3 ml of 0.1 M phosphate buffer (pH 7.4) containing either 1 % Emasol 1130 or 0.5 % cholate) was placed in the 1-cm optical pathway of the Thunberg tube, and the reducer (see below) in the hollow stopper. The tube was then evacuated on a Cenco Hyvac electric pump for 5 min to a pressure below 10 mm Hg, using repeated sharp knocking to expel dissolved O_2. The enzyme was then reduced by inversion of the tube, after which measured amounts of air or of H_2O_2 solution could be added through the evacuation side-arm. The side-arm had a volume of 0.11 ml and by sealing the end with a wet

thumb and opening it momentarily, this volume of air corresponding to 1 μmole of O_2 could be admitted; or by cautious opening, measured volumes of a H_2O_2 solution could be admitted without allowing entry of air. The amount of O_2 contained in the H_2O_2 solution was so small that it could be disregarded. This was shown in a control experiment.

Absorption spectra were recorded by a Perkin-Elmer 350 recording spectrophotometer measuring between 750 and 350 mμ. The wavelength positions were checked on each chart by running a Holmium filter, while correct absorbance was checked with a Chance filter. Slight turbidity was corrected by subtracting the absorbance at 700 mμ which was rarely more than 0.05. Corrections were also made for volume changes due to addition of solutions and where necessary for volume changes due to evacuation.

Reductants

Unless otherwise stated reduction was carried out by dry dithionite diluted with sucrose[12]. An amount of 0.10–0.12 μmole dithionite usually sufficed for full reduction of 0.05 μmole enzyme as indicated by the ratio $A\gamma_{max}(Fe^{2+})/A\gamma_{max}(Fe^{3+}) = 1.26$. A slight excess, 0.2 μmole (4.2 mg dithionite in sucrose corresponding to 0.042 mg $Na_2S_2O_4 \cdot 2H_2O$), was usually added to ensure complete reduction for which a minimum time of 30 min was allowed.

Two other reductants were employed:

(a) catalytic hydrogenation on palladium–asbestos. After H_2 had been replaced by N_2 in order to avoid the formation of H_2O_2 on the catalyst, the catalyst was filtered off anaerobically. Reduction was slow and incomplete and air was permitted to enter during the transfer into the spectrophotometer cuvette.

(b) formamidinosulphinic acid in an evacuated Thunberg tube following the method of SHASHOUA[13]. A 200 molar excess of formamidinosulphinic acid over haem *a* of the enzyme (adding 1 mg to 0.05 μmole of enzyme in 3 ml) did not bring about complete reduction of the oxidase at 21°. The rate of reduction was greatly accelerated by raising the temperature to 40°, or by increasing the formamidinosulphinic acid concentration by a factor of 20. However, under these conditions "modification" of the oxidase occurred as shown by the shift of the Soret band of the ferrous compound from 443 mμ towards 439 mμ (ref. 14). In later experiments with Dr. GILMOUR (unpublished) it was found that a molar excess of formamidinosulphinic acid of 1000 (instead of 200 or 4000) achieves practically complete reduction at 21° in about 30 min with very little "modification" before 60 min.

RESULTS

Formation of the "oxygenated" compound after reduction with dithionite

After evacuation and complete reduction of the enzyme by dithionite, the tube was opened and shaken in air and the spectrum was recorded 1 min later (Curve 3 of Fig. 1). In a number of experiments, the Soret peak was found at 425.9–427.6 mμ, average 427.0 ± 0.6 mμ. The difference in the position of the γ bands of the "oxygenated" compound (in Table I designated "FeO_2") and the original ferric enzyme spectrum was therefore 8–9.5 mμ for the ferric enzyme with a Soret band at 418–420 mμ; it was smaller, 3–5 mμ, for the ferric enzyme with a position of the band at about

422 mμ. It was also smaller on incomplete oxygenation (see below). It was found of interest to compare the ratios of absorbances at the α and γ peaks (γ/α ratio), although this is not reliable during rapid alteration of the spectrum, since approx. 1.5 min elapses between the recording of the two peaks. This γ/α ratio was 8.35–8.65 for the

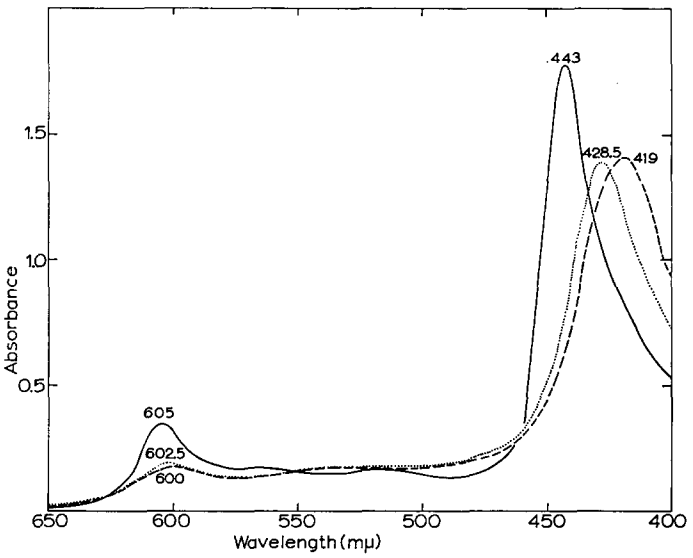

Fig. 1. Absorption spectra of ferrous, ferric and "oxygenated" cytochrome oxidase. Interrupted line, ferric oxidase. Full line, ferrous oxidase after complete reduction by dithionite. Dotted line, "oxygenated" compound after complete oxygenation by molecular O_2 or by H_2O_2.

original ferric enzyme, 5.1 for the ferrous enzyme, and 7.15–7.95 for the fully "oxygenated" enzyme (Table I). The time lapse would tend to shift the Soret band of this compound slightly to a lower wavelength and increase the γ/α ratio, owing to the slow reconversion into the ferric compound. This assumes that no excess of dithionite remains to cause reduction. The Soret peak of the compound was almost equal in height to that of the ferric enzyme, A_γ(compound)/$A_\gamma(Fe^{3+})$ being 0.96–1.0. When, however, a limited amount of O_2 in air was admitted to the tube *e.g.*, 1 μmole of O_2, the γ peak was found at slightly lower wavelengths, the γ/α ratio was below 7.0 and the ratio A_γ("FeO_2")/$A_\gamma(Fe^{3+})$ was also lowered. The increase of absorption at 443 mμ ($A_{443m\mu}/A_\gamma$("FeO_2") > 0.53) (*cf.* Table I) indicated that some ferrous enzyme had not become oxygenated. Where this was very marked (Expts. 494, 471 and 459 of Table I), the position of the Soret band was again at a higher wavelength (427 mμ), but the ratio A_γ("FeO_2")/$A_\gamma(Fe^{3+})$ remained low. This position is probably due to the effect of the absorption of the ferrous enzyme. Under such conditions there was a slow gradual alteration of the spectrum indicating conversion to the ferrous, not to the ferric enzyme. This is not found when the compound is formed with an excess of air, when presumably all dithionite as well as any reducing compound arising from its autoxidation has been removed.

Part of the excess of molecular O_2 over that theoretically required for conversion of the ferrous enzyme to the oxygenated one must be ascribed to excess dithionite

TABLE I

REACTION OF DITHIONITE-REDUCED OXIDASE WITH MOLECULAR O_2 (AIR)

0.05 μmole haem *a* in 3 ml.

Expt. No.	$\gamma_{max}(Fe^{3+})$ ($m\mu$)	$\frac{\mu moles\ O_2}{0.2\ \mu mole\ Na_2S_2O_4}$	$\gamma_{max}(\text{"}FeO_2\text{"})$ ($m\mu$)	γ/α *ratio of* "FeO_2"	$\gamma(\text{"}FeO_2\text{"})/\gamma(Fe^{3+})$ *ratio*	$A_{443m\mu}/\gamma(\text{"}FeO_2\text{"})$ *ratio*
520	418.0	Excess air	427.6	7.24	0.99	0.53
522	419.6	Excess air	427.3	7.14	0.99	0.53
534	420.9	Excess air	427.2	7.60	0.98	0.52
533	420.5	Excess air	427.0	7.95	0.97	0.48
527	418.0	Excess air	425.9	7.51	0.96	0.51
519	418.4	3	426.0	6.96	0.94	0.52
521	419.8	2	426.5	6.76	0.93	0.60
491	422.6	1	425.6	6.49	0.86	0.64
492	422.7	1	425.0	6.76	0.88	0.60
494	423.0	1	427.0	5.92	0.83	0.73
471	422.0	1	427.0	5.39	0.87	—
459	422.5	0.83	427.5	6.55	0.88	0.70

used for the reduction of the enzyme. In an attempt to decrease the amount of O_2 required, formaldehyde (to a final concentration of 10 mM) was added to the reduced enzyme in the evacuated Thunberg tube (*cf.* ref. 15). However, the addition of 0.5 μmole of O_2 after formaldehyde treatment failed to cause transformation into the "oxygenated" compound. This was the same as in the experiments without formaldehyde. Formaldehyde-treated dithionite failed to reduce ferric oxidase. Formaldehyde caused only a gradual slight destruction of the enzyme decreasing the Soret band of the ferric enzyme by 2.3 % in 16 min and 4 % in 95 min. There was a more noticeable effect of formaldehyde on the ferrous oxidase spectrum, decreasing the α and γ bands by about 10 %, and increasing $A_{418m\mu}$ by 7 %. This effect is not due to a "modification" of the enzyme[14], as it is not accompanied by a shift of the band positions but appears rather akin to the formation of non-reducible enzyme.

In some experiments in which insufficient dithionite had been added to achieve complete reduction of the enzyme to the ferrous state, subsequent oxygenation formed the "oxygenated" compound with some admixture of ferric enzyme. The Soret peak was then at 425.7 mμ, the γ/α ratio 7.28, A_γ("FeO_2")/$A_\gamma(Fe^{3+})$ 0.93 and $A_{\gamma,443m\mu}/A_\gamma$("$FeO_2$") 0.53. The circular movement of the Soret band found by LEMBERG AND MANSLEY[1] when insufficient time for reduction was allowed, was not found when the amount of dithionite was limiting. It now appears likely that the circular movement was caused by slow interaction of ferricytochrome a_3 with ferrocytochrome a and the subsequent autoxidation of ferrocytochrome a_3 resulting in the formation of the oxygenated compound. When the amount of reductant, not the time of reduction was limiting, sufficient ferrocytochrome a_3 was formed to react at once with O_2, though some ferric enzyme persists.

As has been reported previously, on standing the Soret peak of the "oxygenated" oxidase moved gradually to shorter wavelengths, *i.e.* in the direction of the peak of the ferric enzyme. The rate of this spontaneous movement varied considerably, *e.g.* in one experiment the peak moved from 427.0 to 423 mμ in 95 min and remained there for a further 190 min; in another experiment it did not move beyond 425.9 mμ, while in a third it reached 422.5 mμ, the position of the ferric band of this particular enzyme, in 160 min. Since the γ/α ratio of the ferric enzyme is higher than that of the "oxygenated" form, one would expect this ratio to increase on standing and this has frequently been observed.

Addition of dithionite autoxidation products to ferrous oxidase

A solution of sodium dithionite containing 0.24–0.96 μmole in 0.1 ml of water, *i.e.* up to 5 times the amount used for the reduction of the oxidase, was shaken with air and later 0.1 ml of this solution was added to the ferrous enzyme in an evacuated Thunberg tube. In some of the experiments autoxidation was carried on until all dithionite had become oxidized as indicated by the disappearance of the absorbance difference at 350 and 380 mμ; in others autoxidation was intentionally left incomplete according to the same criterion. In neither case did addition of the autoxidation products to ferrous oxidase bring about the formation of the "oxygenated" compound. This confirms similar findings of ORII AND OKUNUKI[3] and of WAINIO[5]. It should be noted, however, that there was a time lag of at least 10 sec between the end of aeration and the addition of the products to the ferrous oxidase.

Formation of the "oxygenated" compound from oxidase reduced by reductants other than dithionite

By catalytic hydrogenation followed by aeration after replacement of H_2 by N_2 (see METHODS) the Soret band of the oxidase was shifted from 418 to 425 mμ. The end product was a mixture of predominantly "oxygenated" compound with some ferric oxidase which had not become reduced in the catalytic hydrogenation.

In the experiments in which reduction of the oxidase by formamidinosulphinic acid had been incomplete (formamidinosulphinic acid/haem $a = 200$) the position of the Soret peak after oxygenation was found at 425.5 mμ while in the experiment in which "modification" occurred at 40° with a shift of the Soret band of the ferrous enzyme to 437 mμ, the band was at 423.5 mμ. In the experiment with a greater excess of formamidinosulphinic acid (formamidinosulphinic acid/haem $a = 4000$), the band position after oxygenation was 429 mμ, but again the position of the Soret band of the ferrous enzyme at 438 mμ indicated "modification" during the reduction. In a later experiment of Dr. M. GILMOUR (unpublished) complete reduction was achieved without modification in about 0.5 h; after oxygenation the band was found at 428 mμ.

Formation of the compound with H_2O_2

H_2O_2 in 0.1 ml of a dilute solution was added through the side-arm of the Thunberg tube to the oxidase previously reduced by 0.2 μmole of dithionite. The amount of H_2O_2 required for the formation of the compound was somewhat variable (Table II). Somewhat less than the molar amount of dithionite used to reduce the oxidase (corresponding to 3 times the molar amount of the enzyme) always sufficed for a complete conversion to the 428-mμ compound. In two experiments (No. 473 and No. 523) one-third of this amount of H_2O_2 brought about an almost complete conversion, but in several other experiments this amount was insufficient and most of the enzyme remained in the ferrous state. As has been shown above, the molar amount of atmospheric O_2 which had to be added to the gas phase to bring about conversion into the compound was up to seven times higher. In all other respects, however, the effects of O_2 and H_2O_2 addition were remarkably similar as a comparison of Tables I and II shows. The slight difference in the wavelength position of the maximum (428.5 mμ with H_2O_2, 427.5 mμ with O_2) is hardly significant; as the tables show this is affected by the degree of completeness of conversion, and also by the properties of the enzyme preparation. While a ferric enzyme with a Soret band at 418–419 mμ formed a compound with a maximum at 428 mμ, a ferric enzyme with a band at 422–423 mμ formed a compound with a maximum at 426.4 mμ. The α peak was always found between 600 and 602 mμ. In experiments in which insufficient H_2O_2 was added the ratio $\gamma(\text{"FeO}_2\text{"})/\gamma(Fe^{3+})$ was much smaller than 1, the γ/α ratio of the compound lower than 7, and the ratio $A_{443\text{m}\mu}/\gamma(\text{"FeO}_2\text{"})$ increased as in similar experiments with oxygenation. The last experiment of Table II (No. 517) shows that where the oxidation remained very incomplete further partial reduction to the ferrous oxidase occurred on standing with a shift of the band to longer wavelengths (429.3 mμ) while oxygenation thereafter shifted it back to 427.6 mμ.

Alterations which occur on allowing the compound produced by H_2O_2 to stand were also those observed with the "oxygenated" compound. The Soret band slowly moved to shorter wavelengths, but rarely proceeded beyond 423–424 mμ, even when

TABLE II

REACTION OF DITHIONITE-REDUCED OXIDASE WITH H_2O_2

0.05 μmole haem *a* in 3 ml.

Expt. No.	$\gamma_{max}(Fe^{3+})$ (mμ)	$\frac{\mu moles\ H_2O_2}{0.2\ \mu moles\ Na_2S_2O_4}$	$\gamma_{max}(\text{"}FeO_2\text{"})$ (mμ)	γ/α *ratio of* "FeO_2"	$\gamma(\text{"}FeO_2\text{"})/\gamma(Fe^{3+})$ *ratio*	$A_{443m\mu}/\gamma(\text{"}FeO_2\text{"})$ *ratio*
526	418.0	0.17	428.3	7.10	0.98	0.54
535	418.7	0.15	428.5	7.59	0.98	0.49
524	418.4	0.15	428.5	7.14	1.00	0.53
490	422.8	0.14*	427.0	6.75	0.85	0.53
486	421.2	0.10**	426.0	6.54	0.86	0.61
487	421.0	0.10**	426.0	6.45	0.86	0.61
525	418.5	0.10	427.0	6.62	0.91	0.61
489	422.5	0.09**	426.5	6.43	0.85	0.56
488	422.5	0.07**	425.5	6.47	0.87	0.60
473	422.0	0.05	426.5	7.26	0.96	0.47
423	419.7	0.05	426.8	7.35	—	0.55
517	418.3	0.08	427.6	6.80	0.83	0.64
		after 20 min	429.3	5.80	0.78	0.80
		oxygenated	427.6	7.95	0.87	0.49

* 0.05 + 0.05 + 0.04; 0.10 insufficient.
** 0.05 insufficient.

the original band position of the ferric oxidase had been 418 mμ. The γ/α ratio rose on standing, but the absolute height of the Soret band sometimes decreased, whereas it should increase on the reformation of the ferric enzyme. This is due to a destruction of the oxidase, which varies from a few per cent up to 20 %. After renewed reduction of the compound with dithionite, the absorbance both at 443 mμ and at 604 mμ were lower than those of the original ferrous enzyme (after corrections for dilutions and turbidity) and this decrease was proportionally the same as that of the γ band before reduction. The half-times for the shift from the γ maximum of the H_2O_2 compound to 418 mμ (for the enzyme whose ferric peak was at 418 mμ) varied between 17 and 43 min.

Isosbestic points

The isosbestic point between the 428-mμ compound (initial curve of the completely formed compound) and the 418-mμ ferric enzyme was observed at 424.8 (± 0.5) mμ after oxygenation, and at 425.3 (± 0.3) mμ after treatment with H_2O_2. The isosbestic points with the ferrous enzyme were at 433.4 and 433.1 mμ, respectively. The differences are not significant.

Reaction of the ferrous enzyme with insufficient amounts of O_2 and H_2O_2

In experiments in which an insufficient amount of O_2 or H_2O_2 had been added to the ferrous oxidase, particularly in experiments in which the oxidant did not suffice to shift the Soret band maximum from the position of the ferrous enzyme at 443 mμ, it was observed that the decrease of absorbance at 443 mμ was only about 3–4 times the decrease of the absorbance of the α band at 604 mμ. In a complete reaction the ratio $\Delta A_{443m\mu}/\Delta A_{604m\mu}$ was about 7, while in the stage at which the shift of the lowered maximum at 443 mμ to the 428-mμ maximum occurred, this ratio could be as high as 8–19 (Table III).

TABLE III

REACTION OF DITHIONITE-REDUCED OXIDASE WITH INSUFFICIENT OXIDANT

Oxidant	*Expt. No.*	$\gamma_{max}(Fe^{3+})$ (mμ)	$\Delta A_{443m\mu}/\Delta A_{604m\mu}$		
			*initial**	*later***	*final***
O_2	457	419	2.88	—	6.8
O_2	519	418.5	2.76	7.9	6.5
O_2	484	421.0	3.15	12.3	7.9
H_2O_2	486	421.2	4.34	8.4	6.9
H_2O_2	490	422.8	3.36	19.0	6.4

* γ_{max} = 443 mμ.
** γ_{max} = 426–428 mμ.

Interpretation of these observations is made difficult by the time lag between the measurements at 604 and 443 mμ (about 90 sec later for the latter) because of the reduction of the enzyme to the ferrous state by traces of dithionite left or by reducing products of incomplete dithionite autoxidation. These are shown by the alteration of the spectrum occurring on standing. In one experiment with H_2O_2 this subsequent reduction was virtually complete in 5 min; such a reduction occurring during the time

lag between the measurements at 604 and 443 mμ would obviously decrease the apparent fall of $A_{443m\mu}$ and the measured $\Delta A_{443m\mu}/\Delta A_{604m\mu}$ ratio thus becomes too small. The same subsequent reduction was observed in Expt. No. 457 in the course of 10 min standing after incomplete oxygenation. It is not clear as yet, however, whether the phenomenon illustrated in Table III can be explained on this basis alone; this will be discussed later.

Reactions of the 428-mμ compound

No difference was found in any of the following reactions between the compound formed by oxygenation, or by H_2O_2 addition.

Reduction by dithionite. Addition of an excess of dithionite restored the spectrum of ferrous oxidase. While in some experiments (see above) there was some indication for a destruction of the enzyme during the reaction, in a later experiment by Dr. M. GILMOUR (unpublished) oxygenation and reduction by dithionite could be carried out twice in succession without any alteration of the absorption curve of the ferrous enzyme or loss of its absorbance.

Evacuation. After formation of the compound in two experiments with O_2 (1–3 μmoles O_2 per 0.05 μmole haem *a* and 0.2 μmole dithionite) and in one with H_2O_2 (0.5 μmole H_2O_2 per 0.05 μmole haem *a* and 0.1 μmole dithionite) the Thunberg cuvette was re-evacuated by a high-vacuum pump as described under METHODS, and then allowed to stand for 80 min. In none of these experiments was ferrous oxidase restored by the evacuation, except that in the experiment with 1 μmole O_2 there was a slight increase at 443 mμ, though the maximum remained at 427 mμ. In the other experiments there was no increase at 443 mμ and the position of the γ band moved slowly towards shorter wavelengths and the position of the γ band of the ferric enzyme.

Ferricyanide. Potassium ferricyanide (0.22 μmole in 0.1 ml of water) was added to a solution of enzyme ($\gamma(Fe^{3+}) = 418$ mμ) containing 0.05 μmole haem *a*, in one experiment after oxygenation, in a second one after addition of H_2O_2. There was no immediate alteration of the Soret band position which slowly moved towards shorter wavelengths as it did in the absence of ferricyanide. However, the α band was slightly decreased and shifted by 2–3 mμ to 600 mμ, so that the γ/α ratio was slightly increased. In an experiment with another enzyme preparation ($\gamma(Fe^{3+}) = 422$ mμ) these alterations were somewhat more marked.

Carbon monoxide. O_2-free CO (see METHODS) was bubbled for 5 min into the solution containing the "oxygenated" compound. This time was chosen as a reasonable compromise allowing time for the conversion of any oxygenated compound into a carboxy compound without allowing sufficient time for any reduction of the compound similar to the reduction of the ferric enzyme into its CO compound observed by TZAGOLOFF AND WHARTON[16]. In the α peak region there was a very slight rise of absorbance and a fall, not a rise, in the ratio $A_{590m\mu}/A_{603m\mu}$; a rise would be expected to accompany a transformation of cytochrome a_3 into its CO compound. Alterations of the γ band cannot be expected to be distinct in so far as the γ band of the "oxygenated" compound and that of the CO compound of the oxidase lie at a similar wavelength. On standing under CO the position of the γ band remained almost stationary while LEMBERG AND MANSLEY[1] had found it to move towards shorter wavelengths with a rate close to that on standing in air. Probably under CO the slow spontaneous conversion of the 428-mμ compound into the ferric enzyme is counter-

balanced by a slow reduction of the latter and conversion into the CO compound. The rate of the conversion of the ferric enzyme into the CO compound in the absence of added reducer was slower with our preparation than that found by TZAGOLOFF AND WHARTON[16] and probably varies from preparation to preparation. The effect of CO on the compound formed by 0.15 μmole of H_2O_2 per 0.05 μmole haem *a* was similar; here there was a noticeable movement of the γ band on standing to shorter wavelengths; its rate was slowed down from 22 min to 50 min for the movement of half the distance, but this is inside the range of variation in the absence of CO.

Catalase. Addition of catalase (1.7 μmoles) to the compound formed with O_2 or dithionite did not appear to affect the rate at which the Soret peak moved to shorter wavelengths on standing; the half-time remained within the limits of the movement in the absence of catalase. In one experiment in which only partial conversion had been achieved by H_2O_2, the rate of movement of the Soret band was, however, increased by the addition of catalase.

In an experiment in which catalase was added to the ferrous enzyme before oxygenation, addition of 1 μmole of O_2 which is usually sufficient to cause oxygenation failed to do so. The absorbance of both α and γ peaks was decreased, but the spectrum and the γ/α ratio (5.16) remained those of the ferrous enzyme. However, addition of a second mole of O_2 produced the oxygenated compound with a sharp Soret band at 425 mμ and a γ/α ratio of 6.4, indicating almost complete oxygenation.

DISCUSSION

Table IV shows that the preparation of the "oxygenated" cytochrome oxidase obtained in the experiments described in this paper has an absorption spectrum similar to that previously reported by other workers. However, the height of its Soret maximum at 428 mμ is higher than that previously reported and is practically equal to that of the ferric oxidase at 418 mμ. This finally excludes the possibility of interpreting the spectrum as that of a mixture of ferrous and ferric oxidase. The Soret peak was recorded 3 min after admitting air or H_2O_2 to the reduced enzyme. WAINIO[5] quotes experiments of DAVISON in which rapid scanning immediately after the

TABLE IV

COMPARISON OF "OXYGENATED" COMPOUNDS OF CYTOCHROME OXIDASE

Preparation	γ_{max} (mμ)	$A("FeO_2")/A(Fe^{3+})$ *ratio*	*"Reduced character" of α band** (%)	*"Reduced character" at 443 mμ*** (%)
LEMBERG AND STANBURY (this paper)	426–428	0.95–1.0	7–18	11
LEMBERG AND MANSLEY[1]	427–429.5	0.87	10–23	10–16
ORII AND OKUNUKI[2]	426–428	0.85	22	10
ORII AND OKUNUKI[15]	426.5	0.95	20	15
WAINIO[5]	426–430	0.89	25	10

* $\dfrac{A_{605\,m\mu}("FeO_2")\ minus\ A_{605\,m\mu}(Fe^{3+})}{A_{605\,m\mu}(Fe^{2+})\ minus\ A_{605\,m\mu}(Fe^{3+})}$

** $\dfrac{A_{443\,m\mu}("FeO_2")\ minus\ A_{443\,m\mu}(Fe^{3+})}{A_{443\,m\mu}(Fe^{2+})\ minus\ A_{443\,m\mu}(Fe^{3+})}$

addition of O_2 showed the Soret peak at 430 mμ which then moved to 425–427 mμ within 2–5 min, followed by slow migration to shorter wavelengths. The true position of the peak might thus be at 430 mμ. However, in the present paper the shift of the Soret band to shorter wavelengths on standing has been found slower than previously recorded[1,2,5], and it has been found that incomplete oxygenation with persistence of some ferrous oxidase may cause a slight shift of the band to longer wavelengths. Therefore, 428 mμ probably represents the true position of this band.

Compared with the isosbestic point of ferrous and ferric oxidase ($a + a_3$, see LEMBERG AND MANSLEY[12]) at 432 mμ the isosbestic point of the ferrous and the "oxygenated" oxidase lies at 433–433.5 mμ and has a distinctly higher absorbance (see Fig. 1). The isosbestic point of ferric and "oxygenated" oxidase is here found at 425 mμ, different from those found by ORII AND OKUNUKI[2,15] at 426–428 mμ and by LEMBERG AND MANSLEY[1] at 428.5 mμ.

As in the previous work from this laboratory[1] we have been unable to discover spectroscopic differences between the products of oxygenation and H_2O_2 oxidation. This does not necessarily prove the two products to be identical. However, they have also been found indistinguishable in their stability and in reactions with a variety of reagents (ferricyanide, catalase, CO, low pressure). Variations in the amount of oxidant needed for complete formation of the 428-mμ compound are probably due to differences in the completeness of removal of air from the Thunberg tubes by evacuation with resulting changes in the excess of dithionite. To form the compound, smaller amounts of H_2O_2 (added in solution) are required than of molecular O_2 (added as air to the gas phase). A large part, if not all, of this is certainly due to the comparatively low concentration of O_2 in the solution. Neglecting the small amounts of oxidant required for the final conversion of ferric *via* ferrous to "oxygenated" oxidase, and assuming that 1 mole of dithionite required 1 mole of O_2 for its oxidation, the excess of 0.5–0.8 μmole O_2 added to the gas phase causes an O_2 concentration of about 2 μM in the solution. This is calculated from the volume of air added, its 80 times dilution by the vacuum in the cell, and its equilibration with the 3 ml of fluid in the cell. This concentration is still higher than the K_m of cytochrome oxidase of heart muscle reported as $2.4 \cdot 10^{-8}$ M according to LONGMUIR[17] but is in reasonable agreement with the findings of SEKUZU[18] that 5 % of O_2 in N_2, corresponding to about 60 μM O_2 in the aqueous phase, is needed for maximal activity of the oxidase. Addition of formaldehyde did not markedly decrease the amount of atmospheric O_2 required, although the data of Table IV indicate that ORII AND OKUNUKI[15] achieved a somewhat more complete transformation into the "oxygenated" compound by the use of formaldehyde. In their experiments there was, however, a far greater excess of dithionite than in experiments of the present paper. Either the amount of O_2 required to destroy excess dithionite was in our experiments negligible compared with the effect of distribution of O_2 between gas and aqueous phase, or O_2 was also used up in a reaction with the formaldehyde–dithionite compound in preference to its reaction with the ferrous oxidase. The stoichiometry of the reduction of ferric oxidase and of the transformation of the ferrous oxidase into the 428-mμ compound requires further study.

One might be tempted to explain the apparent difference in the requirements for O_2 and H_2O_2 by assuming that the real reagent in both instances is H_2O_2 which might be formed by the autoxidation of dithionite. However, this assumption is

disproved by other observations. Dithionite can be replaced by reagents which are not likely to form H_2O_2 *e.g.* by formamidinosulphinic acid, or by 1 mole of ferrocytochrome *c* per 1 mole of oxidase-haem *a* (see ref. 19). In agreement with findings of ORII AND OKUNUKI[15] and of WAINIO[5] and DAVISON AND WAINIO[6], ferrous oxidase cannot be oxidized by the addition of products of dithionite autoxidation. This may not be conclusive in so far as there is a time lag of a few seconds between autoxidation of dithionite and the addition of the products to the ferrous enzyme; during this time H_2O_2 may disappear in rapid by-reactions with other products of the autoxidation. On the other hand, it has never been conclusively demonstrated that the oxidative processes in the conversion of haemoglobin to methaemoglobin or choleglobin in the presence of dithionite (DALZIEL AND O'BRIEN[20,21]) are, in fact, due to H_2O_2. They may result from a direct hydrogenation of oxyhaemoglobin by the reductant under certain conditions resulting in the formation of peroxidic compounds.

The spectrum of completely formed 428-mμ compound, in particular the low "reduced character" of the 443-mμ absorption (see Table IV), indicates that no ferrocytochrome *a* is present in the oxygenated compound[1]. The completely oxygenated or H_2O_2-oxidized 428-mμ compound is not converted into ferrous oxidase by evacuation, although evidence for partial reduction after incomplete oxygenation or H_2O_2 oxidation has been found (Tables I and II). The results of DAVISON AND WAINIO[6] who found formation of ferrous enzyme on evacuation may be similarly explained as due to the persistence of reducing substances in incompletely oxidized dithionite, or to the presence of reducing impurities in the oxidase preparation. The latter is the more likely explanation for the very slow rate at which CO reacts with ferric oxidase. With our preparation this was considerably slower (unpublished experiments) than that reported by TZAGOLOFF AND WHARTON[16]. Such a slow reduction explains the effect of CO on the movement of the γ band of the 428-mμ compound to shorter wavelengths on standing. In the present experiments we have found a slowing down of this change by CO, while LEMBERG AND MANSLEY[1] had found no difference. WAINIO[5] had found a slight shift to 430 mμ, but no concomitant increase of the α band, particularly at 590 mμ, which should be the most noticeable effect if there was more than a trace formation of a CO compound by replacement of reversibly bound O_2 by CO.

Ferricyanide (4.5 moles per haem *a*) did not accelerate the rate of spontaneous shift of the 428-mμ band, in contrast to observations of WAINIO[5]. In agreement with observations of ORII AND OKUNUKI[15], the α band was slightly decreased by ferricyanide. The effect was little marked in our experiments, but ORII AND OKUNUKI used a much larger (200 molar) excess of ferricyanide over haem *a*.

None of these results support the assumption that the 428-mμ compound is a reversibly oxygenated compound of cytochrome a_3 similar to oxyhaemoglobin or oxymyoglobin. It rather resembles compounds recently obtained by the reaction of dithionite-reduced horse-radish peroxidase with atmospheric O_2 (YAMAZAKI and co-workers[22–24], WITTENBERG (personal communication)). They also are not reconverted to ferrous peroxidase by evacuation, and do not react rapidly with CO or ferricyanide. They therefore cannot be similar to the "compound III" obtained from metmyoglobin with H_2O_2, at least not if this can be identified with oxymyoglobin (KEILIN AND HARTREE[25]). The present confusion in this field (see ref. 24) shows that further studies are required, which may well tend to diminish the sharpness of division between oxidases and peroxidases.

Addition of catalase to ferrous oxidase before oxygenation failed to prevent the formation of the "oxygenated" compound, although the band was found at 425 mμ and about twice as much air was needed to produce the band than in the absence of catalase. In our earlier experiments[1] we had observed a broad band at 419–425 mμ, which resembled that obtained by ORII AND OKUNUKI[3] in the action of much larger amounts of H_2O_2 (0.1–1 mM) on ferric oxidase; these authors have shown that ferric cytochrome oxidase has itself catalatic activity. In contrast to the reactions of ferrous oxidase with atmospheric O_2 or with a small excess of H_2O_2, this reaction is accompanied by a considerable destruction of the oxidase with loss of absorption. In contrast to our earlier findings, and in agreement with WAINIO[5], catalase did not accelerate the spontaneous shift of the 428-mμ band towards shorter wavelengths.

The low $\Delta\gamma/\alpha$ ratio observed in the reaction of ferrous oxidase with insufficient amounts of O_2 as well as H_2O_2 (Table III) is an interesting phenomenon. It has been interpreted under RESULTS as due to changes occurring during the time lag of recording the α and γ bands and due to slow gradual reduction following incomplete oxygenation. (Note that such an explanation cannot be given to explain the different $\Delta\gamma/\alpha$ ratios found on partial reduction of ferric to ferrous cytochrome oxidase, which are due to the faster reduction of ferricytochrome a by dithionite than of ferricytochrome a_3.) To explain the phenomenon on partial oxygenation as due to different $\Delta\gamma/\alpha$ ratios of cytochromes a and a_3, parallel to partial reduction, would raise great difficulties. Firstly, the compound finally formed has been shown to be distinct from ferric oxidase and to involve oxidation of both ferrous cytochromes a and a_3. One would then have to assume that in incomplete oxidations ferric, not "oxygenated" cytochrome oxidase is formed. This might be possible if the "oxygenated" oxidase, probably a peroxidic compound, rapidly reacts with ferrous oxidase to reform ferric oxidase (although this makes special assumptions necessary to explain the formation of the "oxygenated" compound in complete oxygenation). The low $\Delta\gamma/\alpha$ ratio could then be due to oxidation of ferrocytochrome a prior to that of ferrocytochrome a_3. As cytochrome a_3, not cytochrome a is autoxidizable, this is unlikely. If one tries to overcome this difficulty by assuming with GIBSON AND GREENWOOD[26] that at very low O_2 pressure the oxidation of ferrocytochrome a_3 by molecular O_2 may lag behind that of the reduction of ferricytochrome a_3 by ferrocytochrome a, one faces another contradiction. One would then have to assume a very rapid interaction between ferricytochrome a_3 and ferrocytochrome a which certainly does not occur with purified oxidase preparations. As long as the possibility of an artifact of recording as discussed above has not been excluded, it does not appear profitable to speculate further on the phenomenon.

On standing of cytochrome oxidase preparations at 0–4° for several weeks, the γ band of ferric oxidase is gradually shifted from 418 to 422 mμ. This may be considered in connection with our findings that the γ band of the "oxygenated" compound moves rather rapidly from 428 to 423 mμ, but then much more slowly if at all, to 418 mμ. It appears likely that the slow change of the ferric oxidase on standing involves an oxidation of the protein of the oxidase and that the same reaction accompanies the formation of the "oxygenated" compound. One would then expect a transformation of the "oxygenated" form into the 422-mμ, not the 418-mμ ferric form on standing, while reduction with dithionite would reduce not only the iron but also the protein. Some of the oxidase preparations may contain intrinsic reductants which would cause the slow reconversion of the 422-mμ to the 418-mμ ferric form,

while others may have none. This would also explain the considerable differences in stability of the oxygenated compound which have been found. ORII AND OKUNUKI[15] find some evidence that the enzyme as obtained in the preparation may contain some of "oxygenated" form, but this appears unlikely for the freshly prepared enzyme which has been stood for a few days and has a sharp maximum at 418 mμ.

REFERENCES

1 R. LEMBERG AND G. E. MANSLEY, *Biochim. Biophys. Acta*, 118 (1966) 19.
2 Y. ORII AND K. OKUNUKI, *J. Biochem. Tokyo*, 53 (1963) 489.
3 Y. ORII AND K. OKUNUKI, *J. Biochem. Tokyo*, 54 (1963) 207.
4 K. OKUNUKI, B. HAGIHARA, I. SEKUZU AND T. HORIO, *Proc. Intern. Symp. Enzyme Chem., Tokyo, Kyoto, 1957*, Maruzen, Tokyo, 1958, p. 264.
5 W. W. WAINIO, in T. E. KING, H. S. MASON AND M. MORRISON, *Oxidases and Related Redox Systems*, Vol. 2, Wiley, New York, 1965, p. 622.
6 A. J. DAVISON AND W. W. WAINIO, *Federation Proc.*, 23 (1964) 1322.
7 B. CHANCE, Discussion Remarks to ref. 4.
8 B. CHANCE, Discussion Remarks to ref. 5.
9 G. E. MANSLEY, J. T. STANBURY AND R. LEMBERG, *Biochim. Biophys. Acta*, 113 (1966) 33.
10 Q. H. GIBSON, G. PALMER AND D. C. WHARTON, *J. Biol. Chem.*, 240 (1965) 915.
11 B. F. VAN GELDER, *Biochim. Biophys. Acta*, 118 (1966) 36.
12 R. LEMBERG AND G. E. MANSLEY, *Biochim. Biophys. Acta*, 96 (1965) 187.
13 V. E. SHASHOUA, *Biochemistry*, 3 (1964) 1719.
14 R. LEMBERG AND T. B. G. PILGER, *Proc. Roy. Soc. London, Ser. B* 159 (1964) 436.
15 Y. ORII AND K. OKUNUKI, *J. Biochem. Tokyo*, 57 (1965) 45.
16 A. TZAGOLOFF AND D. C. WHARTON, *J. Biol. Chem.*, 240 (1965) 2628.
17 I. S. LONGMUIR, *Biochem. J.*, 57 (1954) 81.
18 I. SEKUZU, *Ann. Rept. Sci. Works Fac. Sci. Osaka Univ.*, 7 (1959) 147.
19 R. LEMBERG, M. V. GILMOUR AND J. T. STANBURY, *Federation Proc.*, 25 (1966) 2582.
20 K. DALZIEL AND J. R. O'BRIEN, *Biochem. J.*, 67 (1957) 119.
21 K. DALZIEL AND J. R. O'BRIEN, *Biochem. J.*, 67 (1957) 124.
22 I. YAMAZAKI AND L. H. PIETTE, *Biochim. Biophys. Acta*, 77 (1963) 64.
23 I. YAMAZAKI AND K. YOKOTA, *Biochem. Biophys. Res. Commun.*, 19 (1965) 249.
24 I. YAMAZAKI, K. YOKOTA AND R. NAKAJIMA, in T. E. KING, H. S. MASON AND M. MORRISON, *Oxidases and Related Redox Systems*, Vol. 1, Wiley, New York, 1965, p. 485 (in particular p. 499 f. and subsequent discussion).
25 D. KEILIN AND E. F. HARTREE, *Nature*, 166 (1950) 573.
26 Q. H. GIBSON AND C. E. GREENWOOD, *Biochem. J.*, 86 (1965) 541.