Studies of the Oxygenated Compound of Cytochrome Oxidase*

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SUMMARY

Results of rapid reaction experiments indicate that the first observable product formed after the reaction of oxygen with purified reduced cytochrome oxidase is a spectral form which corresponds to that of the oxidized enzyme. This latter is then slowly converted to "The Compound," or so-called oxygenated form. These observations suggest that The Compound is not an intermediate in the cytochrome oxidase-oxygen reaction.

Results of spectrophotometric experiments show that what corresponds to The Compound can be generated after aeration of the enzyme subsequent to reduction with formamidinesulfinic acid, or after aeration of dithionite-reduced oxidase, even when the reduction has been carried out by anaerobic titration to eliminate excess dithionite.

Possible explanations for The Compound are (a) a mixture of ferrous and cupric ions, (b) a mixture of ferrous, ferric, and cupric ions, (c) a higher oxidation state of the heme iron, (d) a change in the oxidation-reduction state of groups on the protein other than iron or copper which affect its absorption spectrum, and (e) a configurational change of the oxidase protein that affects the environment of the heme.

The rates of the reactions occurring when reduced cytochrome oxidase reacts with oxygen have been examined in some detail by Gibson and Greenwood (1-4) and can be accounted for in terms of three consecutive reactions. The first of these, and the most rapid, can be ascribed to the oxidation of reduced cytochrome $a_3$ at a rate of about 30,000 sec$^{-1}$; the second can be accounted for as the oxidation of that copper responsible for the near infrared band at a rate of about 7,000 sec$^{-1}$; the third can be correlated with the oxidation of reduced cytochrome $a$ at a rate of about 700 sec$^{-1}$. Thus, even the slowest of these reactions is quite rapid and can be adequately examined experimentally only by using rapid reaction techniques such as those developed by Gibson and Greenwood (1), Gibson and Milnes (5), and Gibson et al. (6).

Recently, considerable interest has been generated in the significance of the so-called oxygenated form of cytochrome oxidase first described by Okunuki et al. (7) and Sekuzu et al. (8). This form, identified chiefly by means of its Soret maximum at 428 nm, appears following aeration of the reduced oxidase. It has been suggested by Okunuki et al. (7-11) and by Wainio and Davison (12-14) that this form is the result of a complex between oxygen and the enzyme and, therefore, is an intermediate in the reaction of the oxidase. If the so-called oxygenated form is really an intermediate in the reaction with oxygen, then its rate of formation must be at least as rapid as the slowest rate observed in the rapid reaction experiments. Furthermore, a difference spectrum constructed from the changes in absorbance following mixing of oxygen with the reduced enzyme, by means of the flow-flash (1) and stop-flow techniques, should be useful in determining whether the so-called oxygenated complex is formed during the rapid phases of the reaction.

This paper reports the results of experiments which indicate that the so-called oxygenated form is not an intermediate during the oxidation of cytochrome oxidase, but forms much more slowly after the initial oxidation has already occurred. In addition, the results of other experiments are presented in an effort to identify the so-called oxygenated form or The Compound, as it is called by Lemberg (15).

EXPERIMENTAL PROCEDURE

Materials—Cytochrome oxidase was purified from bovine heart mitochondria (16) by the procedure of Griffiths and Wharton (17) or Fowler, Richardson, and Hatefi (18), and from bovine hearts by the method of Yonetani (19). Experiments were carried out in 0.1 M phosphate buffer, pH 7.6, or in 0.1 M Tris-chloride buffer, pH 8.0. Cytochrome $c$ (horse heart, type III) and Tris, purchased from Sigma, were used without further purification. Ascorbic acid, obtained from Merek, was used as the potassium salt. Cholic acid and deoxycholic acid, products of Matheson, Coleman, and Bell, were purified as described by Griffiths and Wharton (17) and used as the potassium salts. Sodium dithionite was obtained from Hardman and Holden, Miles Platting, Manchester, England. Bathophenanthroline was a product of G. Frederick Smith Chemical Company. Formamidinesulfonic acid was purchased from Aldrich.

Flow-flash Experiments—These were made at 25° in the apparatus described by Wharton (17) and used as the potassium salts. Sodium dithionite was obtained from Hardman and Holden, Miles Platting, Manchester, England. Bathophenanthroline was a product of G. Frederick Smith Chemical Company. Formamidinesulfonic acid was purchased from Aldrich.

1 See "Summary."
paratus of Gibson and Greenwood (1) with modifications as
described by Greenwood and Gibson (4).

Stop-flow Measurements—These were carried out at 25° in
the apparatus of Gibson and Milnes (5) in the model manu-
factured by the Durrum Instrument Corporation.

Spectrophotometric Measurements—These were performed at
room temperature with a Cary 14 recording spectrophotometer or
a Beckman DK-2A recording spectrophotometer.

RESULTS

Principal Absorption Spectra of Cytochrome Oxidase—The
absorption spectra of cytochrome oxidase in its oxidized, reduced,
and so-called oxygenated forms are represented in Fig. 1. The
$\alpha$ bands appear at 599, 605, and 600 m$\mu$, respectively, and the
Soret bands at 419, 444, and 428 m$\mu$, respectively. The relative
ratios of absorbance of the Soret bands are 1.00, 1.40, and 1.10,
respectively. In older preparations, the ratio of the last band
decreases and may even become less than 1.00. These observa-
tions are similar to those published previously (7–14, 20), except
that the absorbance of the Soret band of The Compound in the
early reports was always less than that of the oxidized form.
We are unable at present to explain the reason for this difference
if the previous workers used fresh preparations.

The Soret band of the oxidized enzyme has been observed in
different preparations at wave lengths varying from 417 to 424 m$\mu$,
but no significant differences have been found in the specific
activity of these preparations. Although King (21) and we have
observed a shift from 417 m$\mu$ to 424 m$\mu$ on aging preparations
of the oxidase, we have observed that this is not the only instance
when it can be found. At times the Soret band of the oxidized
enzyme has been found at 424 m$\mu$ in fresh preparations purified
from newly prepared bovine heart mitochondria, and at other
times a Soret band has been observed at 418 m$\mu$ in an enzyme
purified from mitochondria that had been stored at −20° for
several days. Lemberg et al. (22) have observed similar anom-
ies. The reason for these changes in the position of the Soret
band of the oxidized enzyme remains unexplained. In any case,
the band always appears at 444 m$\mu$ after reduction of the enzyme
and at 428 m$\mu$ following aeration of the dithionite-reduced
oxidase.

The isosbestic point of the oxidized and the reduced oxidases
is found at 432 m$\mu$; that of the oxidized form and The Compound,
at 423 to 424 m$\mu$; and that of the reduced form and The Com-
pound, at 434 m$\mu$.

Flow-flash Experiments—The difference spectrum presented in
Fig. 2 was determined from data published previously by Green-
wood and Gibson (4) and represents changes of absorbance
occurring 1.5 msec after oxygen had been allowed to react with
the reduced oxidase. This difference spectrum, which has not
been normalized, is quite similar to the difference spectrum of
reduced cytochrome oxidase minus oxidized cytochrome oxidase,
also shown in Fig. 2, and has an identical isosbestic point. It
does not compare as well with the difference spectrum of reduced
cytochrome oxidase minus that of The Compound.

Stop-flow Experiments—Although the stop-flow method does
not offer as fast a time resolution as does the flow-flash technique,
it does have the advantage of using considerably less enzyme per
experiment and it requires somewhat less manipulation. The
slower spectral changes of cytochrome oxidase occurring after
the dithionite-reduced enzyme had been mixed with oxygen were
followed by the stop-flow technique. An example of the type of

record obtained is shown in Fig. 3, which was obtained at 432
m$\mu$, the isosbestic point for the reduced and oxidized forms of
the enzyme. Similarly, records for the spectral changes at 415
m$\mu$ (the wave length of a large negative change in absorbance),
423 m$\mu$ (the isosbestic point of the oxidized form and The
Compound), and 444 m$\mu$ (the wave length of the Soret peak of the
reduced enzyme) are illustrated in Fig. 3, B, C, and D, respect-
ively.

The conclusion drawn from the stop-flow experiments is that,
following the rapid change already mentioned (cf. Fig. 2), most
of which is beyond the time resolution of the stop-flow technique,
there occurs a slower change to a more stable form. If the
FIG. 3. Oscilloscope traces obtained after mixing dithionite-reduced cytochrome oxidase with oxygen-containing buffer in the stop-flow apparatus at 25°. Final concentrations of reactants were: cytochrome oxidase, 5 μM; Tris-chloride buffer, 0.1 M, pH 8.0; deoxycholate, 0.5%. The oxidase was reduced with about 10 mg of sodium dithionite after deoxygenating 20 ml of the solution by flushing with N₂ in a tonometer. Reduction was ascertained by using a hand spectroscope. The concentration of oxygen in the second syringe was 0.29 mm. A, 432 μm; B, 415 μm; C, 423 μm; D, 443 μm.

FIG. 4. Difference spectra of cytochrome oxidase. ---, difference between that formed immediately after mixing the reduced enzyme with oxygen and the stable form assumed secondarily in the stop-flow apparatus; ----, difference between the aerated and the oxidized form observed in the recording spectrophotometer. The concentration of heme a in the former was 5 μM with a 2-cm light path, while in the latter it was 10 μM with a 1-cm light path.

Comparative Rates of Reduction of The Compound and Oxidized Cytochrome Oxidase—To compare the rates of reduction of the oxidized and so-called oxygenated forms by dithionite, experiments were performed in the stop-flow apparatus by mixing the enzyme with a solution containing dithionite (1 mg/10 ml). After mixing, changes in absorbance at a number of wave lengths were determined from the oscilloscope traces. In experiments that almost all the changes related to the two forms could be observed in the stop-flow experiments.

In the particular experiments illustrated here, the elapsed time for the formation of The Compound from the oxidized species was about 60 sec, and the amount of dithionite utilized initially to reduce the enzyme was about 10 mg (60 μmoles)/20 ml of deoxygenated solution. If the amount of dithionite is increased, then the time required for the transition between the two forms decreases, as shown in Table I, suggesting that the reducing agent may be in some way involved in the formation of The Compound.

Even the fastest of these rates is several orders of magnitude slower than the slowest oxidation rate. When dithionite is added in large excess (about 100 mg/20 ml of deoxygenated solution) and the reduced enzyme is mixed with oxygen in the stop-flow apparatus, a reaction record such as shown in Fig. 5 is obtained. This record at 444 μm, along with those observed at other wave lengths, is interpreted as showing that initially there is a change to the oxidized form, followed by a slower change to The Compound, and then a change to the reduced species.

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in which the aerated enzyme was used, the oxidase solution was removed after the stop-flow experiments had been performed and its absorption spectrum was recorded to check that it was still in the form of The Compound.

The rates of reduction of the two forms (oxidized and The Compound) did not differ greatly. The reduction of The Compound showed a lag of 1 or 2 sec, which was not observed with the oxidized form. However, the former required about 60 sec to become 95% reduced, while the latter took about 90 sec to reach the 95% reduced level. The rate of reduction was found to be a function of dithionite concentration, but the relationship between the two forms was constant.

Reaction of Formamidinesulfonic Acid with Cytochrome Oxidase—Some evidence has been presented which connects The Compound with the product of a reaction with peroxide (9, 15, 23, 24). Since the oxidation of formamidinesulfonic acid is not believed to be accompanied by the formation of peroxide, its effect on cytochrome oxidase was investigated. During the course of these studies, Lemberg and Stanbury (15, 20) reported the results of experiments with this reagent which are qualitatively the same as reported here. When 20 mM cytochrome oxidase was allowed to react anaerobically with 4 mM or more of the sulfonic acid at 25°, the absorption spectrum soon corresponded closely to that of the reduced enzyme, with maxima at 604 and 443 mμ. The time required to reduce the oxidase completely was a function of the reagent concentration, taking 1 hour with 20 mM formamidinesulfonic acid and 4 hours with 0.4 mM reagent. After aeration of the reduced samples by gentle shaking in air, the Soret band was observed at 427 to 428 mμ. The a band appeared at 600 mμ, with a slightly higher absorbance than that of the oxidized enzyme. When the reaction was carried out with 0.4 mM formamidinesulfonic acid, the Soret band appeared at 428 mμ upon aeration and then slowly shifted to 424 mμ, the wave length of the starting oxidized enzyme, while the a-band returned to its original position at 599 mμ. At 4 mM and 20 mM formamidinesulfonic acid the Soret band of The Compound did not return to the position of the oxidized form, perhaps because under the conditions of the experiment sufficient sulfonic acid remained active in the solution. When concentrations of reagent below 0.4 mM were used with 20 mM oxidase, the position of the reduced Soret band was located between 424 and 437 mμ. On aeration of these solutions, the Soret band shifted to 427 to 428 mμ if the peak of the reduced enzyme was located at wave lengths greater than 428 mμ. Where the peak of the reduced oxidase was observed between 424 and 428 mμ, then the Soret band of the aerated enzyme did not shift immediately but slowly returned to the position of the oxidized form.

Anaerobic Titration with Sodium Dithionite—A principal reason for suggesting that The Compound is formed by reaction with peroxide is that the oxidation of sodium dithionite by gaseous oxygen is accompanied by the production of hydrogen peroxide. The latter then would presumably be free to react with the enzyme. However, if the oxidase is reduced by dithionite anaerobically so that no excess dithionite is present upon termination of the reduction, then no hydrogen peroxide should form from the dithionite when air is admitted to the enzyme solution. For this experiment a solution of cytochrome oxidase in a cuvette fitted with a rubber serum stopper was made anaerobic by flushing with H2 for 1 hour. The H2 has no observable effect on the enzyme. The anaerobic oxidase was titrated with a solution of dithionite by injecting a hypodermic syringe through the serum cap. The progress of the reduction of the enzyme was followed spectrophotometrically and was terminated just before the enzyme became reduced completely. The reduced enzyme was then aerated by bubbling oxygen through the solution. The absorption spectrum of the aerated sample had a Soret peak at 428 mμ, which then shifted slowly to shorter wave lengths. This shows that The Compound can form even though no excess dithionite is present to generate peroxide.

DISCUSSION

Okunuki et al. (8–11), as well as Wainio and Davison (12–14), have postulated that the spectral form of cytochrome oxidase, most readily recognized by a Soret peak at 428 mμ and observed following aeration of the reduced enzyme, is an intermediate in the reaction sequence of the enzyme and is most likely a complex with oxygen. Thus, they have referred to it as the oxygenated form.

Our findings cast doubt on this hypothesis since we observed, by both the flow-flash and stop-flow rapid reaction techniques, that a spectral form corresponding to that of the original oxidized enzyme appears first, well within 1.5 msec after the reduced enzyme has been mixed with oxygen. This spectral form is followed by a change, occurring over a period of seconds, to a form which corresponds quite well with that of the so-called oxygenated cytochrome oxidase. Since any intermediate formed during the oxidation of the reduced enzyme must have a lifetime of less than 1.5 msec, it is clear that The Compound does not meet this criterion. Pending further counterproposals, we suggest that The Compound or so-called oxygenated form not be considered as an intermediate in the cytochrome oxidase-oxygen reaction. Likewise, during the reductive reaction, we can find no evidence favoring an obligate route through this particular form.

The present experiments present no evidence which conflicts with the suggestion of Greenwood and Gibson (4) that a molecule of O2 enters a functional unit of the oxidase which contains two heme a groups and 2 atoms of copper. There, in consecutive reactions, 4 electrons are transferred from one heme a, 2 atoms of copper, and the second heme a, in that order. It is only after these reactions have been completed that heme a reacts with cytochrome c and initiates the multistep process of reduction of the unit, in which, as shown several years by Gibson et al. (6), reduction of cytochrome a occurs first, in association with reduction of copper (in agreement with the work of van Gelder and Muijsers (25) and van Gelder and Slater (26)), and then is succeeded by the slower reduction of cytochrome a.

Although the present kinetic evidence indicates that The Compound is not an intermediate during the oxidation of cytochrome oxidase, its true identity remains hidden. One fact that does emerge from the data of this communication as well as from the recent experiments of Lemberg and Stanbury (20) is that The Compound, or something very similar to it, can be produced in several ways, some of which are not known to produce peroxide on oxidation. Thus, it may occur following oxidation of the enzyme reduced with dithionite or cytochrome a (9), upon reaction of either oxidized or reduced enzyme with hydrogen peroxide, or after aeration of the oxidase reduced with formamidinesulfonic acid.

The possibilities that The Compound results from partial
reduction of the oxidase (27) or from a separate reduction of part of the iron or copper (12, 15, 28) are not rigorously excluded by the present data. However, the results of Wainio and Davison (12, 13) argue against the former, while the observations of Beinert et al. (29) and of Gilmour (30) suggest that copper returns to the cupric state upon oxidation of the reduced enzyme. The oxidation state of the heme iron under conditions described in this communication is presently under investigation in this laboratory. The possibility must also be entertained that The Compound may result from a conversion of the heme iron to a higher oxidation state. Furthermore, in the absence of quantitative titration data during the reductive and oxidative reactions, it is difficult to eliminate the possibility that groups associated with the enzyme undergo oxidation-reduction changes which are not in unison with those of iron or copper but yet affect the absorption spectrum.

Another possible explanation for The Compound, although there is no direct evidence, is that it results from a conformational change of the protein which affects the environment of the heme. The new conformation, in the absence of further reduction, gradually reverts to the conformation of the original oxidized enzyme. The effect of cytochrome c in accelerating the return of the oxidized form as reported by Orii and Okunuki (9) could be explained by the effect that its binding would have on the conformation of the enzyme. The energy for the initial change could come from that liberated during oxidation of the reduced enzyme. Although this hypothesis will be tested with the technique of circular dichroism, the rather ambiguous results obtained by this method on heme proteins do not provide much encouragement for a clear answer. Thus, the final resolution may require the development of as yet unforeseen methods.

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