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. The 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 affects its absorption spectrum, and (e) a configurational change of the oxidase protein that affects the environment of the heme.

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 mp, 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 Plating, 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 26° in the ap-

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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 manufactured 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 α bands appear at 599, 605, and 600 µm, respectively, and the Soret bands at 419, 444, and 428 µm, 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 observations are similar to those published previously (7-14, 20), except that the absorbance of the Soret band of the Compound in the earlier 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 µm, 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 to 424 µm 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 µm in fresh preparations purified from newly prepared bovine heart mitochondria, and at other times a Soret band has been observed at 418 µm in an enzyme purified from mitochondria that had been stored at -20° for several days. Lemberg et al. (22) have observed similar anomalies. 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 µm after reduction of the enzyme and at 428 µm following aeration of the dithionite-reduced oxidase.

The isosbestic point of the oxidized and the reduced oxidases is found at 432 µm; that of the oxidized form and The Compound, at 423 to 424 µm; and that of the reduced form and The Compound, at 434 µm.

Flow-flash Experiments—The difference spectrum presented in Fig. 2 was determined from data published previously by Greenwood 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 µm, the isosbestic point for the reduced and oxidized forms of the enzyme. Similarly, records for the spectral changes at 415 µm (the wave length of a large negative change in absorbance), 423 µm (the isosbestic point of the oxidized form and The Compound), and 444 µm (the wave length of the Soret peak of the reduced enzyme) are illustrated in Fig. 3, B, C, and D, respectively.

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
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 μmole)/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.

Table I

<table>
<thead>
<tr>
<th>Amount of sodium dithionite added initially</th>
<th>Time required for 90% conversion to The Compound</th>
<th>First order rate constant</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg/ml</td>
<td>sec</td>
<td>sec⁻¹</td>
</tr>
<tr>
<td>0.5</td>
<td>50</td>
<td>0.046</td>
</tr>
<tr>
<td>2.5</td>
<td>12</td>
<td>0.191</td>
</tr>
<tr>
<td>5.0</td>
<td>8</td>
<td>0.272</td>
</tr>
</tbody>
</table>

Fig. 5. Oscilloscope trace obtained at a wave length of 444 μm after mixing dithionite-reduced cytochrome oxidase with oxygen-equilibrated buffer in the stop-flow apparatus. Experimental conditions were the same as those described for Fig. 3, except that about 100 mg of sodium dithionite were added initially to reduce 20 ml of deoxygenated enzyme solution in the tonometer.
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 dichromate concentration, but the relationship
between the two forms was constant.

**Reaction of Formamidinesulfinic 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 formamidinesulfinic 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 qualita-
tively the same as reported here. When 20 μM cytochrome
oxidase was allowed to react anaerobically with 4 mM or more of
the sulfinic acid at 25°, the absorption spectrum soon corre-
sponded closely to that of the reduced enzyme, with maxima at
604 and 443 μM. The time required to reduce the oxidase
completely was a function of the reagent concentration, taking 1
hour with 20 mM formamidinesulfinic 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 μM.
The α band appeared at 600 μM, with a slightly higher absor-
ance than that of the oxidized enzyme. When the reaction was
carried out with 0.4 mM formamidinesulfinic acid, the Soret band
appeared at 428 μM upon aeration and then slowly shifted to
424 μM, the wave length of the starting oxidized enzyme, while
the α-band returned to its original position at 599 μM. At 4 mM
and 20 mM formamidinesulfinic 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
sulfinic acid remained active in the solution. When concentra-
tions of reagent below 0.4 mM were used with 20 μM oxidase,
the position of the reduced Soret band was located between 424 and
437 μM. When reduced samples of these solutions, the Soret band shifted
to 427 to 428 μM if the peak of the reduced enzyme was located at
wave lengths greater than 428 μM. Where the peak of the reduced
oxidase was observed between 424 and 428 μM, 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 μM, 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 μM 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
complex. Our findings cast doubt on this hypothesis since we
observed, by both the flow-flask 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 sug-
gest 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 reduc-
tion 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 c (9), upon reac-
tion of either oxidized or reduced enzyme with hydrogen peroxide,
or after aeration of the oxidase reduced with formamidinesulfinic
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 to 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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REFERENCES

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