Cytochrome c Oxidase Components

V. A CYTOCHROME $a$ PREPARATION FREE OF CYTOCHROME $a_3$*

SHIGEO HORIE† AND MARTIN MORRISON

From the Department of Biochemistry, Medical Research Institute, City of Hope Medical Center, Duarte, California

(Received for publication, August 19, 1963)

Despite considerable spectral evidence supporting the concept of two cytochromes, $a$ and $a_3$, in the terminal respiratory system (1-15), there has not been uniform agreement that the mammalian cytochrome $c$ oxidase system contains these two cytochromes (16-18).

Recent work which distinguished these cytochromes chemically has strengthened the spectral observations (19, 20). It was possible by the use of borohydride under special conditions to alter the prosthetic group of cytochrome $a_3$ without affecting the prosthetic group of cytochrome $a$. The present work is an extension of these observations.

One of the classical methods employed in protein purification procedures is the selective destruction of contaminating proteins. By applying such a procedure, it has been possible to prepare cytochrome $a$ free of cytochrome $a_3$. It is the purpose of this paper to present the method of preparation as well as define some of the properties of a preparation of cytochrome $a$ free of cytochrome $a_3$.

EXPERIMENTAL PROCEDURE

Cytochrome $c$ oxidase was prepared as previously described (8). The preparation, containing 10 mg of protein per ml, was dissolved in ammonium sulfate-cholate-phosphate buffer. This buffer was prepared by dissolving 11.34 g of ammonium sulfate in 100 ml of 0.1 M phosphate buffer, pH 7.4, which contained 1.2% sodium cholate. The pH of the solution was adjusted to 7.4 by adding 4 M ammonium hydroxide.

To 40 ml of this solution, 36 mg of solid sodium dithionite were added. The preparation was stirred gently for 1 minute and was then made 0.015 M with respect to cyanide by the addition of a freshly prepared solution of 1 M sodium cyanide. The solution was allowed to stand for a few minutes, and 40 mg of sodium borohydride were added with gentle stirring for 1 minute. The temperature was maintained at 0-4° during the preceding and all succeeding steps.

The solution was allowed to stand for 1 hour and then, with occasional stirring, for another ½ hour. At the end of this time, about 12 mg of sodium dithionite were added, followed by 1.62 g of ammonium sulfate. The solution was stirred for 15 minutes and centrifuged at 15,000 $\times$ g for 15 minutes. The precipitate was discarded, and 1.42 g of ammonium sulfate were added to the supernatant. The mixture was stirred for 5 minutes and centrifuged at 15,000 $\times$ g for 5 minutes.

The precipitate was dissolved in 25 ml of ammonium sulfate-phosphate buffer and centrifuged at 15,000 $\times$ g for 15 minutes. To the supernatant solution were added 10 mg of sodium dithionite and 7.69 g of ammonium sulfate per 100 ml of solution. The suspension was stirred for 5 minutes and centrifuged at 15,000 $\times$ g for 5 minutes.

The precipitate was dissolved in 20 ml of 0.1 M phosphate buffer pH 7.4, containing 1% Tween 80, and centrifuged at 15,000 $\times$ g for 30 minutes to remove all turbidity. The preparation thus obtained can be stored at $-10^\circ$.

Enzyme activity was assayed by spectrophotometric determination of the rate of oxidation of reduced cytochrome $c$ (8, 21). The protein (8, 22), heme $a$ (8, 23), and copper (24) were determined as previously described. The spectra were obtained by the use of the Beckman DK-2 recording spectrophotometer.

RESULTS

Fig. 1 shows a comparison of the spectra of a cytochrome $c$ oxidase preparation and of a cytochrome $a$ preparation free of cytochrome $a_3$. A number of differences can be observed.

One of the most significant points is that saturation with carbon monoxide does not produce a significant shift in the spectrum of the reduced cytochrome $a$ preparation. This is in sharp contrast to the effect of carbon monoxide on the spectrum of reduced cytochrome $c$ oxidase.

The position of the absorption maxima in the cytochrome $a$ preparation is at 600 mp in the visible region and at 439 mp in the Soret region. Both of these maxima are removed about 3 mp from the maxima observed in the preparation containing both cytochromes $a$ and $a_3$. The $\beta$-maximum is also more apparent in the cytochrome $a$ preparation. Last, the ratio of the extinctions of the $\gamma$- and $\alpha$-maxima ($\gamma:\alpha$) is very close to 4 in the cytochrome $a$ preparation, and is about 5 in the cytochrome $c$ oxidase preparation.

All of these results are consistent with previous spectral evidence (8, 9) on the properties of cytochrome $a$ present in cytochrome $c$ oxidase.

Table I presents the absorption maxima of both the absolute and difference spectra, as well as the extinction coefficient of cytochrome $a$. As Fig. 1 shows, there is only a slight change in the spectrum of cytochrome $a$ on saturation of the reduced preparation with carbon monoxide. The Soret peak shifts slightly toward a shorter wave length, and there is a slight decrease in the extinction. This may be the result of the presence of some denatured material, but neither additional treatment with cyanide and borohydride nor ammonium sulfate fractionation is effective in removing this material.

The position and extinction of the oxidized cytochrome are
Fig. 1. Comparison of spectra of cytochrome c oxidase and cytochrome a. A, spectrum of reduced cytochrome c oxidase and carbon monoxide complex. ——, cytochrome c oxidase reduced with dithionite; ----, the same solution saturated with carbon monoxide. B, spectrum of cytochrome a preparation. ——, a preparation of cytochrome a reduced with dithionite; ----, the same solution above saturated with carbon monoxide.

Table I
Spectral properties of cytochrome a
The data were obtained by the methods described in the text.

<table>
<thead>
<tr>
<th>Spectrum</th>
<th>( \lambda_{max} ) (nm)</th>
<th>Absorbance index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absolute spectra</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oxidized*</td>
<td>425</td>
<td>75.7</td>
</tr>
<tr>
<td>Reduced</td>
<td>439</td>
<td>94.1</td>
</tr>
<tr>
<td>Difference spectra (reduced minus oxidized)</td>
<td>600</td>
<td>36.9</td>
</tr>
<tr>
<td>( \gamma )-Peak</td>
<td></td>
<td>542</td>
</tr>
<tr>
<td>( \alpha )-Peak</td>
<td></td>
<td>103</td>
</tr>
</tbody>
</table>

* Autoxidized for at least 30 minutes at room temperature.
† Based on heme a content.

Table II
Enzyme activity
The method of enzyme assay is that described in text. The assay was performed at 25°C.

<table>
<thead>
<tr>
<th>Compound</th>
<th>( k ) (sec(^{-1})/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytochrome c oxidase</td>
<td>4.05</td>
</tr>
<tr>
<td>Cytochrome a</td>
<td>0.058</td>
</tr>
</tbody>
</table>

preparation with the activity of the starting cytochrome c oxidase. The results clearly show the great loss in activity caused by removal of cytochrome \( a_3 \). The cytochrome \( a \) preparation has less than 2% of the activity of the starting material. Repeating the procedure used for the removal of cytochrome \( a_3 \) does not significantly decrease the enzyme activity.

In Table III, the effect of cytochrome \( a \) on the activity of cytochrome c oxidase is shown. It is apparent from these results that cytochrome \( a \) neither enhances nor inhibits the rate of oxidation of cytochrome c catalyzed by cytochrome c oxidase.

Discussion
Much of the support for the concept that cytochrome c oxidase contains a single hemoprotein, "cytochrome \( a \)," has come from
en the cytochrome c oxidase preparation.

or the reverse, has never been achieved (16-18). This report

is the rate of oxidation of cytochrome c.

respectively, in Experiments 1, 2, and 3.

in Experiment 3.

3.66 /~g in the assay cuvette for Experiments 1 and

1440

4.13 lower than 5.1, the value of the preparation which contains

a and a3 in the visible region, but much lower than the value given

for this discrepancy will be published more fully.

for such a preparation in the Soret region. Thus, the ratio of

extinction of the hemochromogen. Our present value for the

extinction of reduced cytochrome c oxidase is 21.2. The reasons

are identical for the isolated cytochrome a and the cytochrome a

oxidase preparation (9). The positions of the absorption maxima

described in this paper has spectral properties consistent in every

chemical identities.

tinguishable, but also that they are distinct and independent

positions of the absorption maxima to that of the Soret maxima is identical in the

both cytochrome a and a3. This ratio of the extinction of the

visible to the Soret extinction coefficient of cytochrome a is

the cytochrome a oxidase, does not combine with either carbon

monoxide or cyanide. Again, the preparation

nally defined (1). The cytochrome a preparation, in contrast to

carbon monoxide such as cyanide or carbon monoxide. Hence, the method employed in obtaining

the extinction coefficient of the cytochrome a preparation is 22.8 and 94.1, respectively. The preparation

contains approximately an equal molar ratio of copper to heme.

The preparation has a very low cytochrome c oxidase activity. Cytochrome a is reduced by cytochrome c, but cytochrome a does

not enhance the activity of the cytochrome c oxidase preparation. This may simply reflect the fact that the

rate-limiting steps in the over-all activity of our cytochrome c oxidase preparation only involved cytochrome a.

Since cytochrome a has previously been shown to be distinguishable from cytochrome a3 spectrally (8) and chemically (19, 20), the fact

that cytochrome a can now be isolated free of cytochrome a3 clearly indicates that these two cytochromes are not identical.

SUMMARY

Starting with a purified preparation of mammalian cytochrome c oxidase containing cytochromes a and a3, a method is described for a preparation which has the properties of cytochrome a free of cytochrome a3. The absorption maxima of reduced cytochrome a are at 590 and 439 mp, and the millimolar extinction coefficients are 22.8 and 94.1, respectively. The preparation contains approximately an equal molar ratio of copper to heme.

The preparation has a very low cytochrome c oxidase activity. Cytochrome a is reduced by cytochrome c, but cytochrome a does not enhance the activity of the cytochrome c oxidase preparation. This may simply reflect the fact that the rate-limiting steps in the over-all activity of our cytochrome c oxidase preparation only involved cytochrome a.

The preparation has a very low cytochrome c oxidase activity. Cytochrome a is reduced by cytochrome c, but cytochrome a does not enhance the activity of the cytochrome c oxidase preparation. This may simply reflect the fact that the rate-limiting steps in the over-all activity of our cytochrome c oxidase preparation only involved cytochrome a.

Another criterion which distinguishes cytochrome a3 from cytochrome a is the ability of a3 to react with various reagents such as cyanide or carbon monoxide. Again, the preparation described has properties consistent with cytochrome a3 as originally defined (1). The cytochrome a preparation, in contrast to cytochrome c oxidase, does not combine with either carbon monoxide or cyanide.

Further, the preparation is, when compared to the original cytochrome c oxidase, not autoxidizable, although it will slowly catalyze the oxidation of cytochrome c. This may simply reflect

the presence of a small amount of cytochrome a3. Efforts to remove this last bit of activity, such as repeated fractionation and passage through a Sephadex column, have not been successful in removing this activity. In this regard, it should be noted that the preparation of cytochrome a still contains copper, and the ratio of copper to heme remains approximately 1:1 as in the cytochrome c oxidase (24). It may be that the remaining cytochrome c oxidase activity and the slow autoxidizability of the cytochrome a preparation can be attributed to this copper (24).

The cytochrome a preparation is not readily reducible by a soluble succinate-cytochrome c reductase system. When cytochrome c is added to this system, however, the cytochrome a is reduced. The data of Table III show that the cytochrome a preparation does not enhance the activity of the cytochrome c oxidase preparation. This may simply reflect the fact that the rate-limiting steps in the over-all activity of our cytochrome c oxidase preparation only involved cytochrome a.

Since cytochrome a has previously been shown to be distinguishable from cytochrome a3 spectrally (8) and chemically (19, 20), the fact that cytochrome a can now be isolated free of cytochrome a3 clearly indicates that these two cytochromes are not identical.
Shigeo Horie and Martin Morrison

Cytochrome c Oxidase Components: V. A CYTOCHROME a
PREPARATION FREE OF CYTOCHROME a3
Shigeo Horie and Martin Morrison


Access the most updated version of this article at http://www.jbc.org/content/239/5/1438.citation

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/239/5/1438.citation.full.html#ref-list-1