Cytochrome c Oxidase Components

III. SPECTRAL PROPERTIES OF CYTOCHROMES a AND a₃*

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The spectral properties of cytochrome c oxidase have been the subject of a number of studies (1-14). Although these studies have not, in themselves, convinced all investigators that more than one hemoprotein was involved, they have confirmed the original observations which formed the basis for distinguishing the cytochromes a and a₃. These observations by Keilin and Hartree (1) indicated that the absorption at 605 and 444 nm was due to two components. One of these, which they termed cytochrome a₃, was autoxidizable and combined with carbon monoxide and cyanide, causing a spectral shift. The other, cytochrome a, which was not autoxidizable and did not combine with these reagents, showed no spectral alteration.

Some investigators (15-19) have interpreted the spectral data obtained with purified cytochrome c oxidase as indicating that only a single cytochrome is present. Wainio (15, 16) has further suggested that the copper present in the purified preparations may account for the spectral changes observed with carbon monoxide, cyanide, and nitric oxide.

It has been held that only separation of the cytochromes a and a₃ would offer satisfactory evidence for the existence of two cytochromes. However, in any study designed to isolate the cytochromes a and a₃, it would be necessary to correlate the properties of isolated components with the properties of the cytochromes in the intact cytochrome c oxidase system. It is, therefore, essential to establish the properties of the cytochromes while they are still a part of that system. Yonetani (13) has recently made an effort to do this by detailed study of his purified preparation. By the use of difference spectra, he was able to distinguish quantitatively the absorption due to cytochrome a and that due to cytochrome a₃.

In a continuation of our efforts to characterize the components (2, 3, 8, 20) of the cytochrome c oxidase, we have attempted to define further the spectral properties of cytochrome a and cytochrome a₃. With this information, we should have added criteria useful in comparing preparations which contain only cytochrome a or cytochrome a₃.

**EXPERIMENTAL PROCEDURE**

The cytochrome c oxidase was prepared as previously described (2). The heme and protein concentrations were determined as previously reported (2).

In most of the spectral studies, the concentration of cytochrome c oxidase contained 0.0123 mm heme a and 2 mg of protein per ml. The preparation was dissolved in 0.1 M phosphate buffer, pH 7.4, which contained 0.5% Tween 80. In experiments with cyanide, the concentration was 1.5 x 10⁻² M. In order to obtain the spectra of carbon monoxide derivatives, the solution was saturated with carbon monoxide by passing the gas through the solution for at least 3 minutes. The spectra were recorded on a Beckman DK-2 recording spectrophotometer.

The various forms of cytochrome c oxidase that were employed in the spectral studies are described below.

**Oxidized cytochrome c oxidase** (a++, a₃++), was considered to be the enzyme as prepared. The preparation was diluted with 0.1 M phosphate buffer containing 0.5% Tween 80 to the concentration indicated.

**The cyanide derivative of the oxidized preparation** (a++, a₃++ CN), was the solution described above, containing in addition 1.5 x 10⁻² M cyanide.

**Reduced cyanide preparation** (a++, a₃++ CN) was the above preparation to which dithionite was added immediately before the recording of the spectrum.

**Reduced cytochrome c oxidase** (a++, a₃+-) was considered to be the enzyme as prepared. The preparation was diluted with 0.1 M phosphate buffer containing 0.5% Tween 80 to the concentration indicated.

The **cyanide derivative of the reduced preparation** (a++, a₃+- CN), was obtained by adding sufficient cyanide to make the reduced preparation 1.5 x 10⁻² M with respect to cyanide.

The **carbon monoxide derivative of reduced cytochrome c oxidase** (a++, a₃+- CO) was prepared by passing carbon monoxide through the reduced preparation for 3 minutes while the sample was submerged in an ice bath.

In order to record spectra in which the oxidation state of cytochrome c differed from that of cytochrome a₃, the procedure outlined in Table I was employed. Two sets of matched cuvettes were placed in the reference and sample beams of a double beam spectrophotometer. Solutions were prepared as indicated and the spectra shown in the figures are those recorded.

In the difference spectrum shown in Fig. 4, a base line was recorded with the cyanide derivative of the oxidized preparation in the B cuvettes of the reference and sample beam; the oxidized cytochrome c oxidase was placed in the A cuvette of both beams. Dithionite was then added to the A cuvette of this sample beam, and the solution was saturated with carbon monoxide, thus converting the material to the carbon monoxide derivative of reduced cytochrome c oxidase. Just before the spectrum was recorded, dithionite was also added to the B cuvette of the

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1 Purchased from the Nutritional Biochemical Corporation.
### Table I

**Outline of experimental procedures**

<table>
<thead>
<tr>
<th>Figure No.</th>
<th>Reference cuvettes</th>
<th>Sample cuvettes</th>
<th>Algebraic sum of the recorded spectrum (sample and reference)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Reduced cyanide, $a^{++}$, $a_{s}^{++}$CN</td>
<td>Reduced, $a^{++}$, $a_{s}^{++}$</td>
<td>$a^{++} + a_{s}^{++}$</td>
</tr>
<tr>
<td>2</td>
<td>Oxidized cyanide, $a^{+++}$, $a_{s}^{+++}$CN</td>
<td>Oxidized, $a^{+++}$, $a_{s}^{+++}$</td>
<td>$a^{+++} + a_{s}^{+++}$</td>
</tr>
<tr>
<td>3</td>
<td>Reduced cyanide, $a^{++}$, $a_{s}^{++}$CN</td>
<td>Reduced cyanide, $a^{++}$, $a_{s}^{++}$</td>
<td>$a^{++} + a_{s}^{++}$</td>
</tr>
<tr>
<td>4</td>
<td>Oxidized, $a^{+++}$, $a_{s}^{+++}$CN</td>
<td>Reduced cyanide, $a^{++}$, $a_{s}^{++}$CN</td>
<td>$a^{++} + a_{s}^{++}$</td>
</tr>
</tbody>
</table>

### Table II

**Outline of experimental procedures**

<table>
<thead>
<tr>
<th>Figure No.</th>
<th>Percent reduction of cytochromes</th>
<th>Sample cuvettes</th>
<th>Reference cuvettes</th>
<th>Algebraic sum of the recorded spectrum (sample and reference)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>100 0 1</td>
<td>1 Reduced, $a^{++}$, $a_{s}^{++}$</td>
<td>1 Reduced cyanide, $a^{++}$, $a_{s}^{++}$CN</td>
<td>$a^{++} + a_{s}^{++}$</td>
</tr>
<tr>
<td>6</td>
<td>0 100 1</td>
<td>1 Reduced, $a^{++}$, $a_{s}^{++}$</td>
<td>1 Reduced cyanide, $a^{++}$, $a_{s}^{++}$CN</td>
<td>$a^{++} + a_{s}^{++}$</td>
</tr>
<tr>
<td>7</td>
<td>100 100 1</td>
<td>1 Reduced, $a^{++}$, $a_{s}^{++}$</td>
<td>1 Reduced cyanide, $a^{++}$, $a_{s}^{++}$CN</td>
<td>$a^{++} + a_{s}^{++}$</td>
</tr>
</tbody>
</table>

* In this case three cuvettes are employed as indicated.

Reference beam, converting the solution in this cuvette to the reduced cyanide preparation.

By varying the concentration of the cytochrome c oxidase preparation in the cuvettes in the reference and sample compartments, it was possible to obtain spectra of the cytochrome c oxidase preparation equivalent to partially reduced and partially oxidized cytochrome $a$ and cytochrome $a_{s}$. These experiments are outlined in Table II. The concentration of the preparation was varied by accurate dilution with 0.1 M phosphate buffer containing 0.5% Tween 80. In Table II, a dilution factor of 1 indicates no dilution, whereas 1:4 indicates that 1 volume of the preparation was diluted to 4 volumes. Thus, on the assumption that the absorption of the preparation follows Beer's Law, the diluted material will have one-fourth the absorption of the

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*In this case three cuvettes are employed as indicated.*
original material. As shown in Table II, two sets of matched cuvettes were employed in most experiments. In one experiment, as indicated, three sets of cuvettes were employed.

RESULTS

The recorded spectrum is the algebraic sum of the absorbing materials in the system. In Fig. 1, for example, the sample beam contains $a^{++} + a^{++}CN$ in one cuvette and $a^{++} + a^{++}CN$ in the other. The reference beam contains only $a^{++} + a^{++}CN$. The algebraic sum is thus

$$a^{+++} + a^{+++}CN + a^{++} + a^{++} - (a^{++} + a^{+++}CN) = a^{+++} + a^{++}$$

and the recorded spectrum in Fig. 1 is that of oxidized cytochrome $a$ and reduced cytochrome $a_3$.

In a similar manner, the spectrum recorded in Fig. 2 is equivalent to reduced cytochrome $a$ and oxidized cytochrome $a_3$. Fig. 3 presents the spectrum of oxidized cytochrome $a$ and the reduced cytochrome $a_3$-carbon monoxide complex.

In Fig. 1, where the spectrum is equivalent to a mixture in which only cytochrome $a_3$ is reduced, the greatest changes in the spectrum, as compared to the oxidized spectrum, are in the Soret region. The position of the absorption maxima in the visible region is at a slightly higher wavelength (605 mp) than when both cytochromes $a$ and $a_3$ are totally reduced (603 mp). Fig. 4 shows a series of spectra in which the percentage of reduction of cytochrome $a_3$ is varied. The position of the visible absorption maximum is at 605 mp, whereas the Soret peaks are at 424 mp with a shoulder at approximately 442 mp when cytochrome $a_3$ is reduced. When both cytochromes are oxidized, the Soret peaks of the two cytochromes are indistinguishable with a maximum at 421 mp. When cytochrome $a_3$ is reduced, the oxidized Soret peak of cytochrome $a$ is at a longer wave length (423 mp).

Fig. 2 shows the spectrum of reduced cytochrome $a$ and oxidized cytochrome $a_3$. In contrast to the reverse situation as shown in Fig. 1, there is a marked change in the spectrum in both the visible and Soret regions upon reduction of cytochrome $a$. The peak in the visible region is at a slightly shorter wavelength (602 mp) than that obtained in Fig. 1. There is a β-peak clearly present in Fig. 2, with a maximum at 581 mp. The spectral changes, which occur as the cytochrome $a$ is progressively reduced, can be seen in Fig. 6.

The spectra of cytochrome $a_3$-carbon monoxide complex and oxidized cytochrome $a$ are shown in Fig. 3. Also shown is the spectrum of cytochrome $a_3$-carbon monoxide complex in the presence of reduced cytochrome $a$. The position of the absorption maximum is shifted more than 10 mp to 591 mp when cytochrome $a$ is in the oxidized form. Compared to the usual spectrum in which both cytochromes are reduced, the β-peak is also more pronounced and the Soret peak is shifted to shorter wave lengths.

Fig. 4 shows the difference spectra between the oxidized and reduced cytochrome $a_3$ and the carbon monoxide complex. The position of the α-absorption peaks in this spectra is at 605 mp for the reduced and at 584 mp for the carbon monoxide complex.

In both cases, a β-peak can be most clearly seen at 548 mp.
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FIG. 3. The absolute spectra of cytochrome c oxidase containing reduced cytochrome a3-carbon monoxide complex and cytochrome a in reduced and oxidized forms. The conditions which were employed in obtaining the spectrum are outlined in the text and Table I. The extinction values are based on the total heme a content of the preparation. ----, Reduced cytochrome a3-carbon monoxide complex and oxidized cytochrome a; --, reduced cytochrome a3-carbon monoxide complex and reduced cytochrome a.

FIG. 4. Difference spectra of cytochrome a3. The experimental procedures employed in obtaining the spectra are outlined in the text and Table I. ---, The spectrum of reduced cytochrome a3-carbon monoxide complex versus oxidized cytochrome a3; -----, the spectrum of reduced cytochrome a3 versus oxidized cytochrome a3.

FIG. 5. Spectra of cytochrome c oxidase in which the cytochrome a3 component is reduced to varying extents while the cytochrome a component remains completely oxidized. The procedure employed in obtaining the spectra are outlined in the text and Table II. The cytochrome c oxidase employed was 7.1 mM with respect to heme a and contained 1 mg per ml of protein. ---, Cytochrome a3 completely reduced; -----, cytochrome a3 50% reduced; ----, cytochrome a3 0% reduced.

Discussion

It was the object of the present study to produce spectra of a cytochrome c oxidase preparation in which the cytochrome a and cytochrome a3 each existed in a different valence state. In this way, it was hoped to establish that the cytochrome a and a3 were distinct components and, further, to distinguish the spectral properties of each.

The fact that cyanide combined with both reduced and oxidized cytochrome a3, as first indicated by Keilin and Hartree (1),

for the carbon monoxide complex, and although fused with the a-peak, there is clearly a shoulder in the 560 to 570 μm region of the reduced spectrum. In the Soret region, the maxima are at 430.5 μm for the carbon monoxide derivative and 443.5 μm for the reduced compound.

It is clear from Table II that spectra equivalent to partial reduction of either or both cytochromes can be obtained. The spectra are shown in Figs. 5, 6, and 7. In all cases, the absorption maxima and the shape of the curves are in complete agreement with those shown in Figs. 1 and 2.

The relatively small contribution of cytochrome a3 to the absorption at 605 μm, and its relatively large contribution in the Soret region, are apparent in Figs. 1 and 5. In contrast, cytochrome a has a much larger absorption in the visible region and also accounts for much of the absorption at 444 μm in the reduced form. The isosbestic points for the two cytochromes in the Soret region are not greatly separated, occurring at 430 and 462 μm for cytochrome a3, with a very slight shift to a higher wavelength for cytochrome a.
made it possible to produce cytochrome $a_3$ in a different valence state from the cytochrome $a$. Yonetani (13) had suggested that the oxidized cytochrome $a_3$-cyanide complex is not readily reduced, even by dithionite. In the present work, it was observed that the oxidized cyanide complex of cytochrome $a_3$ resisted reduction for some time after the addition of dithionite. Thus, it was possible to obtain a preparation with cytochrome $a$ in the reduced form and still maintain cytochrome $a_3$ in the oxidized form. Advantage was also taken of the fact that the recorded spectrum was the algebraic sum of the absorbing materials in the light path.

Yonetani (13) obtained a series of difference spectra of the cytochromes $a$ and $a_3$. He employed the oxidized cytochrome $a_3$-cyanide complex as oxidized cytochrome $a_3$, in cases where he could assume that the cyanide effect was small, and he corrected by calculation for the effect of cyanide in other experiments. The present study has, in most instances, been able to confirm Yonetani's results, and we have been able to record directly all the difference spectra that he calculated.

Table III records the position of the maxima and the ratio of the extinction of the Soret and $\alpha$-peaks of the difference spectra. The positions of the maxima are in complete agreement, but the ratio of the extinctions is not. The reason for these discrepancies is not apparent, but it is possible that the different techniques used to obtain the spectrum, or differences in the preparation employed, may be involved.

Although Yonetani (13) reported a ratio of 13 for the extinction of the $\alpha$- and Soret maxima in the difference spectrum of reduced and oxidized cytochrome $a_3$, the present work shows this ratio to be significantly higher. In the case of the difference spectrum of reduced and oxidized cytochrome $a_3$, the ratio is lower than that of cytochrome $a_3$ and the value in the present work is lower than the value of 4.5 given by Yonetani (13). It is interesting to note that the reduced versus oxidized difference spectrum of the cytochrome $a_3$-cyanide complex has a Soret to $\alpha$ ratio very similar to that of cytochrome $a$. The cyanide complex of cytochrome $a_3$ also resembles cytochrome $a$ in that it has a $\beta$-peak which is missing from the cytochrome $a_3$ spectra.

Azide can also be employed in the same manner as cyanide, which was used in this paper. Azide forms a complex with both the oxidized and reduced cytochrome $a_3$. The oxidized cytochrome $a_3$-azide complex, like the cyanide complex, is not readily reduced by dithionite. However, the oxidized azide does not appear to be as stable in the presence of dithionite as is the cyanide complex, and is converted to the reduced form more readily. Cyanide is, therefore, the reagent of choice.

Figs. 3 and 4 demonstrate that the cytochrome $a_3$-carbon monoxide complex has the characteristics attributed to the photochemical action spectrum (6, 7, 21). In addition, the spectrum of the reduced cytochrome $a_3$-carbon monoxide complex with oxidized cytochrome $a$ shown in Fig. 3 is identical with that obtained by treatment with ferricyanide of a reduced

<table>
<thead>
<tr>
<th>Difference spectrum</th>
<th>Position maximum</th>
<th>Extinction ratio ($\alpha$/$\beta$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$a_3^{++} - a_3^{+}$</td>
<td>605 (580-570)</td>
<td>443.5</td>
</tr>
<tr>
<td>$a_3^{++}CO - a_3^{++}$</td>
<td>550 (545-548)</td>
<td>430.5</td>
</tr>
<tr>
<td>$a_3^{++}CN - a_3^{+++}CN$</td>
<td>500</td>
<td>514</td>
</tr>
<tr>
<td>$a^{+} - a^{++}$</td>
<td>600 (515-518)</td>
<td>445</td>
</tr>
</tbody>
</table>

Table III

Difference spectra maxima

Fig. 6. Spectra of cytochrome c oxidase in which the cytochrome $a$ component is reduced to varying extents while the cytochrome $aa$ component remains completely oxidized. The experimental procedure is outlined in the text and Table II. The cytochrome c oxidase employed in these experiments was 1 mg per ml with respect to protein and 7.1 $\mu$M with respect to heme $a$.

Fig. 7. Spectra of cytochrome c oxidase in which cytochrome $a$ and $aa$ are reduced to varying extents. The cytochrome c oxidase employed was 1 mg per ml with respect to protein and 7.1 $\mu$M with respect to heme $a$. 

$\alpha$: Cytochrome $a$ completely reduced; $\beta$: cytochrome $a$ 75% reduced; $\gamma$: cytochrome $a$ 50% reduced; $\delta$: cytochrome $a$ 25% reduced; $\epsilon$: cytochrome $a$ 0% reduced.

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cytochrome c oxidase preparation saturated with carbon monoxide (2). The absolute spectra of the cytochromes a and a3 make apparent a number of points that are not evident in the difference spectrum. First, the positions of the a-peaks of cytochromes a and a3 are not identical, but are separated by about 3 mp. Second, cytochrome a accounts for much more of the absorption of cytochrome c oxidase than has been suggested by previous workers (4, 5, 9-12, 22). Thus, in the Soret region, the absolute spectrum suggests that cytochrome a accounts for at least twice as much absorption as cytochrome a3.

The data bring into question the basic assumptions of a number of these previous investigations (4, 5, 9-12, 22). It has been usual to assume that the value obtained in the difference spectrum between 605 and 630 mp is all attributed to cytochrome a3. In Table IV, the actual percentage reductions of the cytochromes a and a3 are compared with the percentage reductions of the preparation in each region of the spectrum as determined by the methods of Smith (12) and Chance (22-26). The discrepancies are apparent. Attention has already been called to the need for a correction (18), although the magnitude of the proposed correction is not completely in agreement with the data presented in Table IV. This may be the result of the different preparations employed.

Williams (27) has indicated that the difference in the positions of the Soret maxima of the reduced and oxidized forms of a hemoprotein is a guide to the type of heme complex. A large difference in the position of the Soret implies a high spin or ionic complex. According to this theory, the sum of the magnetic moments of the ferrous and ferric forms is directly related to the change in the Soret maximum between the two forms. Since the Soret maxima of the cytochromes a and a3 are very nearly identical, this postulate would lead to the prediction that both of these cytochromes have the same ionic character. The cyanide derivatives of cytochrome a3, on the other hand, have absorption maxima which are closer together, indicating a lower magnetic moment even than cytochrome a. The actual magnetic susceptibility data do not appear to correlate with these speculations and a re-evaluation of the observations and theory is undoubtedly required.

Numerous investigators have made a number of assumptions concerning the spectra of the cytochromes a and a3 (4, 5, 9-11, 22-29) and on this basis speculated on the molar relationship between the two cytochromes. The spectral data presented here clearly indicate that the original assumptions were for the most part faulty. A direct analysis for the molecular ratio of the two cytochromes should now be possible (3) and our efforts are being directed toward this goal.

### Table IV

<table>
<thead>
<tr>
<th>Actual reduction</th>
<th>Calculated reduction*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytochrome a</td>
<td>Cytochrome a3</td>
</tr>
<tr>
<td></td>
<td>605 to 630 mp</td>
</tr>
<tr>
<td>0%</td>
<td>100%</td>
</tr>
<tr>
<td>0%</td>
<td>50%</td>
</tr>
<tr>
<td>50%</td>
<td>50%</td>
</tr>
<tr>
<td>50%</td>
<td>0%</td>
</tr>
<tr>
<td>100%</td>
<td>0%</td>
</tr>
</tbody>
</table>

* Calculated on basis of difference spectra peak to trough values at wave length indicated (22).

The spectral properties of a purified cytochrome c oxidase preparation have been investigated. A procedure is outlined by which the difference spectra of any desired combination of reduced and oxidized cytochromes a and a3 can be obtained. The reduced versus oxidized difference spectrum of cytochrome a3 and the difference spectrum of oxidized versus reduced carbon monoxide-cytochrome a3 are presented.

The procedure can be modified to obtain absolute spectra of the cytochrome c oxidase preparation in which cytochromes a and a3 are present in different valence states. Thus, spectra are shown of the preparation with either oxidized cytochrome a and reduced cytochrome a3 or, conversely, reduced cytochrome a and oxidized cytochrome a3. It is also possible to obtain the spectrum of the preparation equivalent to partially reduced cytochrome a3 in the presence of oxidized cytochrome a or, conversely, the equivalent of partially reduced cytochrome a in the presence of oxidized cytochrome a3. Examples are also given of the spectra of various combinations of partially reduced cytochromes a and a3.

It is noted that most of the previous speculations concerning the contributions of the two cytochromes to the absorption at 444 and 605 mp in the reduced form are questionable. The most significant point in this regard is the high contribution of cytochrome a to the Soret peak of the reduced cytochrome c oxidase preparation. Cytochrome a accounts for at least twice as much of the absorption at 444 mp as does cytochrome a3.

**Acknowledgment**—The heart muscle used in this study was generously supplied by the Farmer John Packing Company, Vernon, California.

### REFERENCES

Cytochrome c Oxidase Components: III. SPECTRAL PROPERTIES OF CYTOCHROMES a AND a3
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