Selective Reduction of Cytochrome c Oxidase with Borohydride*

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Since the pioneer studies of Keilin and Hartree (1), considerable evidence has accumulated indicating that cytochrome c oxidase contains two heme moieties that react differently; hence the designation cytochrome a-a. Reasons for this designation include kinetic studies that show (a) differences in the rate of reduction and oxidation of cytochrome c oxidase measured at the a versus b absorption maxima (2-5), (b) rates of reduction measured at 444 mµ, which indicate the presence of two reducing components (4), and (c) rates of reduction in the presence of CO, which indicate the presence of a fast (444 mµ) and a slow (430 mµ) reducing component (6).

Recently, Morrison and Horie (7, 8) reported the reduction of the carbonyl group on only the cytochrome a-heme by pretreatment of the oxidase with borohydride. The cytochrome a-heme was not affected, suggesting the selective reducing ability of borohydride toward cytochrome a.

It is the purpose of this paper to show that, contrary to other reports (7, 9, 10), borohydride can reduce the iron of the hemes of cytochrome c oxidase. The reduction of iron can be accomplished with or without the concomitant reduction of the carbonyl group of the cytochrome a-heme. In addition, it is shown that the carbonyl group of cytochrome a was reduced by borohydride only after incubation at alkaline pH.

EXPERIMENTAL PROCEDURE

Cytochrome c oxidase was prepared by a modification of the procedure of Smith and Stotz (11). The principal change in the procedure was the use of Tris buffer, which permitted greater ease of extraction of the cytochromes and sharper fractionation of the hemoproteins by ammonium sulfate. All operations were carried out at 0-4°. Ground pig heart (400 g) was washed successively with 3 liters each of distilled water, 0.9% NaCl, and distilled water. Each wash lasted approximately 20 minutes. A phosphate extract was prepared by blending in a Waring Blendor (11) the washed tissue with 750 ml of 0.1 M phosphate buffer, pH 7.4, for 6 to 7 minutes followed by centrifugation at 2,500 x g for 20 minutes. The supernatant fluid was saved, and the precipitate was re-extracted with 600 ml of buffer. Acetic acid (3 M) was added to the pooled extracts to adjust the pH to 5.5. The mixture was centrifuged for 30 minutes at 18,000 x g, and the supernatant was discarded. A Tris suspension of the precipitate was prepared by adding 0.1 M buffer, pH 7.4, to a final volume of 600 ml and blending the mixture briefly in a Waring Blender. The Tris suspension was incubated for 1 hour with 150 mg of trypsin and 12 ml of 40% sodium cholate. The end of this incubation, 156 g of (NH₄)₂SO₄ were added and the mixture was stirred for 20 minutes prior to centrifugation for 20 minutes at 12,000 x g. The precipitate was discarded, and 36 g of (NH₄)₂SO₄ were added to the supernatant. After 20 minutes, the mixture was centrifuged as above. To the supernatant fluid, 30 g of (NH₄)₂SO₄ were added and the mixture was again centrifuged. The colorless supernatant was discarded, and the precipitate of cytochrome c oxidase was dissolved in the Tris buffer and frozen. Approximately 425 mg of oxidase (based on dialyzed dry weight) were obtained.

All spectra were obtained with a Perkin-Elmer 400A spectrophotometer. All reagents were added directly to the cuvettes before the spectra were recorded. CO was added by bubbling the gas through the contents of the cuvette for the indicated times. Either solid sodium borohydride or a solution of borohydride in 0.1 N NaOH was added, as indicated, to the cuvettes.

RESULTS

Enzymatic properties of the cytochrome c oxidase preparation are summarized in Table 1. It can be seen that only on the addition of cytochrome c is oxygen consumed. In the presence of cytochrome c, oxygen is not consumed, even when the concentration of oxidase has been increased 37-fold. Likewise, as shown in Fig. 1, the cytochrome c oxidase preparation oxidized reduced cytochrome c only on the addition of cytochrome c.

Reduction of cytochrome c oxidase by borohydride is shown in Figs. 2 and 3, and is compared with reduction obtained by the use of dithionite. It is apparent that essentially all the iron of the oxidase heme is reduced soon after the direct addition of considerable borohydride. There is little or no evidence of carbonyl reduction by borohydride as revealed by the similarity of the spectra in the a region obtained with the two reducing agents, as well as by the lack of the characteristic 420 or 417 mµ absorption maxima for the reduced and the CO complex, respectively, of the reduced carbonyl component (8-13).

Once it was established that borohydride could reduce the iron of both hemes, a mixture of borohydride and dithionite was subsequently used for investigation of the reduction of the carbonyl group. This permitted a decrease in the concentration of boro-

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1 Trypsin, Grade I-300, was obtained from Nutritional Biochemicals Corporation.

2 This precipitate was rich in cytochromes b and c.

1402
hydride used, and ensured anaerobic conditions through the use of dithionite. Dithionite itself was without effect on the carbonyl group.

As shown in Fig. 4, reduction of the carbonyl group depended upon the concentration of borohydride used and the time allowed. At a low concentration of borohydride (3.5 mg), a normal reduced spectrum was obtained initially, although with time further reduction was obtained. Increased amounts of borohydride decreased the time required for the reduction to occur. With 15 mg of borohydride, complete reduction was obtained after 65 minutes, although essentially all the reduction was completed by 35 minutes. It will be noted also that absorbance at 440 μs was not altered by the different concentrations of borohydride used. (Compare the — with O—— O.) The absorption maximum for the borohydride-reduced CO complex was at 417 μm, in agreement with the results of other workers (8, 13). The borohydride-reduced CO complex was obtained only if the final pH of the reaction mixture was between 8 and 9.5.

Total reduction of the carbonyl groups by borohydride, as evidenced by the disappearance of absorption at 440 μm and the appearance of a single absorption maximum at 417 μm, was obtained provided that the final pH was greater than 9.5. These results are shown in Fig. 5 and are compared with the spectra of the oxidase reduced with dithionite and flushed with CO.

### Table I

**Oxygen uptake by cytochrome c oxidase**

<table>
<thead>
<tr>
<th>Additions</th>
<th>Oxygen uptake</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
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<tr>
<td>Cytochrome c, 20 μm moles</td>
<td>81</td>
</tr>
<tr>
<td>Cytochrome c, 26 μm moles</td>
<td>0</td>
</tr>
<tr>
<td>None*</td>
<td>0</td>
</tr>
<tr>
<td>Cytochrome c, 26 μm moles</td>
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</tr>
</tbody>
</table>

* Cytochrome c oxidase concentration was increased to 1.8 mg.

**Fig. 2 (left).** Absorption spectra of reduced cytochrome c oxidase. The solid line (——) represents the oxidase reduced with solid sodium dithionite, and the dashed line (—), the oxidase reduced with solid sodium borohydride. Identical spectra were obtained in the a-band region. The cuvettes contained 3.9 mg of cytochrome c oxidase in a volume of 3 ml. The oxidase was diluted with 0.1 M Tris buffer, pH 7.4, containing 1% cholate. Borohydride (approximately 10 mg) was added directly to the cuvette, and the spectrum was recorded after 10 minutes. The final pH of the reaction mixture reduced with borohydride was 7.7.

**Fig. 3 (right).** Absorption spectra of the reduced CO complex of cytochrome c oxidase. The solid line (——) represents the oxidase reduced with solid sodium dithionite and flushed with CO for 2 minutes. The dashed line (—) represents the oxidase flushed with CO for 10 minutes and then reduced with 10 mg of solid sodium borohydride for 10 minutes. Identical spectra were obtained in the a-band region. The cuvettes contained 2.8 mg of oxidase in a volume of 3 ml. The oxidase was diluted with 0.1 M Tris buffer, pH 7.4, containing 1% cholate. The final pH of the mixture reduced with borohydride was 7.7.

**DISCUSSION**

Cytochrome c oxidase is readily reduced by dithionite or cysteine, both of which are autoxidizable reducing agents, or by reduced cytochrome c. It is not completely reduced by phenylendiamine, ascorbate, or hydroquinone.

In the present study, reduction of the oxidase by borohydride was investigated under conditions in which oxygen had been removed. Dissolved oxygen was removed either by displacement with CO, which also meant that any reduced cytochrome a would be trapped as a stable CO complex, or by hydrogen liberated from the borohydride. Under both conditions, borohydride readily reduced cytochrome c oxidase. If, indeed, oxygen was present in the system, only a partial reduction of the oxidase was achieved. The spectra then were similar to those obtained with phenylendiamine-ascorbate mixture as the reducing agent.

To establish that borohydride reduction of the oxidase was not dependent on endogenous cytochrome c, manometric and spectrophotometric assays were performed. In each instance a requirement for added cytochrome c was shown, establishing the absence of endogenous cytochrome c in the oxidase preparation. It has been shown that borohydride can reduce the iron of heme a, resulting in maxima at 430 and 587 μm for the pyridine-hemochromogen, and it is not surprising that the hemoprotein also can be reduced. It has not been established whether both the cytochrome a and a₂ portions of cytochrome c oxidase are directly reduced by borohydride, or whether only one is reduced and in

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3 A. P. Martin, unpublished data.
In the present study, total reduction was achieved after incubation of the oxidase with alkali. Actually, the effect of 0.1 N NaOH was investigated to determine whether induced changes in protein conformation would affect the reactivity of the oxidase. It is apparent that in the presence of alkali geometry about the heme of cytochrome a has been altered. Since borohydride has been used also to reduce disulfide groups, it is possible that cleavage of these bonds, as well as alterations in electrostatic and hydrophilic properties due to alkali and cholate, allows for completeness of reaction of the oxidase and accounts for our results.

Maximum reduction of the a1 portion of cytochrome c oxidase was obtained even in the absence of CO. These results differ from those reported by Morrison and Horie (8), who found that cyanide apparently was required to obtain the borohydride effect. Since the heme of cytochrome a1 readily combines with CO and the carbonyl group is easily reduced by borohydride, this heme must be accessible. In contrast, the heme of cytochrome a must be inaccessible.

The results presented are consistent with the designation of a cytochrome a and a,1cytochrome a designating the portion of the oxidase with the heme that is easily reduced by borohydride and cytochrome a designating the portion with the heme that is reduced with difficulty. Nevertheless, it is still impossible to indicate whether cytochrome c oxidase represents one or more hemoproteins.

**SUMMARY**

The reduction of cytochrome c oxidase by borohydride has been investigated. The reduction of the iron and carbonyl groups of the heme by the direct addition of borohydride to the cuvette has been demonstrated. Carbonyl reduction was favored at more alkaline conditions. The carbonyl of cytochrome a1 heme was readily reduced by borohydride, whereas that of cytochrome a heme was reduced only after previous incubation with sodium hydroxide. These results are interpreted as indicating a difference in geometry about the hemes.

**REFERENCES**

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