Spectral Characteristics and Interconversions of the Reduced, Oxidized, and Oxygenated Forms of Purified Cytochrome o*

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SUMMARY

Cytochrome o can be chemically reduced by excess dithionite and oxidized by ferricyanide and ammonium persulfate. The latter reagent is colorless and allows spectral observations in the Soret region. The spectrum of the "oxygenated" form of the reduced cytochrome, which has absorption maxima at 576, 543, and 414 nm, is elicited in aerobic solution by enzymatic reduction with NADH, and when formed in this way the oxygenated form is very stable. When the solution is made anaerobic, the spectrum of the reduced (non-oxygenated) form appeared. The reduced form becomes spectrophotometrically detectable when the dissolved oxygen falls below 14 μM and is at half-maximal concentration at 6 μM oxygen. The reintroduction of oxygen results in reappearance of the spectrum of the oxygenated form. The addition of ammonium persulfate or sodium dithionite to the oxygenated form produced the oxidized or reduced cytochrome, respectively. Bubbling carbon monoxide through solutions of the oxygenated form shifted the absorption maxima to 566, 535, and 419 nm, which are the absorption maxima characteristic of the reduced cytochrome-carbon monoxide complex. Cyanide, which can bind to the oxidized form of cytochrome o, had no effect on the spectrum of the oxygenated form, but the formation of this form from oxidized cytochrome o and NADH was inhibited in the presence of cyanide. Hydrogen peroxide forms a complex with cytochrome o that has a spectrum similar to the spectrum of the oxygenated form.

It is obvious that a heme protein, such as hemoglobin, whose primary physiological function is oxygen transport, would have an "oxygenated" form, but the existence of oxygenated forms of other types of heme proteins has recently been established. Okunuki and his co-workers (1, 2) first reported the appearance of an intermediate oxygenated form of cytochrome a (their terminology for cytochrome oxidase). Their interpretation of the data was disputed at first; the objections were raised that their results were due to denatured cytochrome or a mixture of reduced and oxidized forms, since the Soret band of the oxygenated form was located at a wavelength between the Soret bands for the oxidized and reduced forms of the cytochrome (3). More recent work, however, has confirmed the existence of an oxygenated form of cytochrome oxidase, which is distinct from the oxidized (ferric) and the reduced (ferrous) forms (4, 5).

Evidence for the existence of an oxygenated form of another terminal oxidase, cytochrome o, has been presented in the accompanying report (6). Cytochrome o has been spectrally identified primarily in prokaryotes and has been purified from Vitreoscilla (6, 7), which is a filamentous myxobacterium, that lacks both a- and c-type cytochromes (8, 9). The reduction of purified cytochrome o from Vitreoscilla by NADH is catalyzed by component(s) present in purified preparations of the cytochrome (6, 10). The first spectrophotometrically detectable product of this reduction in aerobic solutions has absorption maxima at 577, 544, and 420 nm as revealed by (NADH + cytochrome o) minus (cytochrome o) difference spectra. Since this spectrum appeared only on the presence of a reducing agent (NADH) and oxygen, and because of its resemblance to the spectrum of oxyhemoglobin, it was postulated that this spectrum was due to the appearance of an oxygenated form of reduced cytochrome o (6). In this paper we present more evidence for this postulate and more detailed spectral studies on cytochrome o, with particular heed to the interconversions between the oxidized, reduced, and oxygenated forms of the cytochrome. The reduced form of cytochrome o in this report, will mean the ferrous, non-oxygenated form, whereas the oxygenated form will mean that form which arises by the addition of molecular oxygen to the ferrous form, no attempt being made to define the nature of the bonding.

MATERIALS AND METHODS

The growth of Vitreoscilla sp., Murray strain 389, and the purification of cytochrome o from frozen cells of this strain of Vitreoscilla have been described in the accompanying report (6). Fractions of cytochrome o from the final step of this purification procedure, a DEAE-cellulose chromatography, having an A415/A345 > 1.0 were pooled and used for the spectral studies unless otherwise indicated in the legends. The penultimate step, a Sephadex G-100 chromatography, separated cytochrome o from a high molecular weight heme pigment, which was not reducible by NADH and possessed high catalase activity and has been tentatively identified as a catalase (6). Since this pigment was not reducible by NADH, spectral changes observed on addition of NADH to less purified preparations of cytochrome o can be ascribed to the cytochrome. The less pure cytochrome o preparation of the 45 to 65% ammonium sulfate precipitate purification stage exhibited high NADH oxidase activity, capable of essentially completely deoxygenating the solution, and this fraction...
was used when transition from the oxygenated cytochrome to the reduced form, was being observed.

All spectra were performed at room temperature with a Zeiss DMR 21 recording spectrophotometer in cuvettes of 1-cm light path. Zeiss MT-2 microcuvettes containing 0.2 ml were used for many experiments. Chemical reduction or oxidation of the cytochrome was performed by the addition of a few grains of sodium dithionite or ammonium persulfate, respectively, to the solution in the cuvette. Simultaneous measurements of oxygen uptake and absorbance changes were performed by cutting a piece of styrofoam to fit into the cell chamber of a Hitachi spectrophotometer and hold a disposable test tube (16 × 150 mm) in the light path. The oxygen electrodes fit snugly into the tube with the tip of the probe just above the light path. The solution in the light path (4 ml) was stirred with a small magnetic stirring bar driven from below with an air-powered magnetic stirrer. Oxygen uptake was measured with a YSI model 55 oxygen analyzer with a Beckman recorder.

An Hitachi Perkin-Elmer model 139 spectrophotometer with a Sargent model SRL recorder was used to study the deoxygenation of cytochrome o solutions and estimate the affinity of the cytochrome for oxygen. These reactions were followed at 435 nm after the addition of an appropriate volume of 0.02 M NADH. To accelerate the removal of oxygen, aliquots of cell-free extracts of Vitreoscilla were added in some experiments. These cell-free extracts were prepared from cells grown for 4 days at room temperature in 500 ml of medium and harvested by low-speed centrifugation. After suspending in 10 ml of 0.1 M sodium phosphate (pH 7.5), the cells were sonicated with a MSE 100-watt ultrasonic disintegrator at maximum setting twice for 1 min at 0°C with a 1-min interval between to avoid overheating. The cell suspension was centrifuged 10 min at 2000 × g at 4°C in a Sorvall RC 2 refrigerated centrifuge and the resultant supernatant was used for the enzyme assays. Protein content of the cell-free extracts was determined by the biuret method, that of the purified cytochrome o fractions by Am, assuming 1.0 A4280 = 1 mg of protein per ml.

RESULTS

To study the characteristics of the three known forms of cytochrome o, oxidized, reduced, and oxygenated, and transitions between them, it was necessary first to obtain spectra of solutions of cytochrome o containing only the pure form of each. When Orii and Okunuki (11) treated their preparations of cytochrome a with ferriyanide, a widely used reagent for oxidizing cytochrome oxidase, they observed spectral changes indicating the presence of small amounts of reduced cytochrome a; consequently, they defined ferriyanide-treated cytochrome a as the completely oxidized form. When preparations of purified cytochrome o were treated with ferriyanide, absorbancy decreases at 577 and 544 nm were observed. Since ferriyanide absorbs light below 500 nm, its usefulness as a chemical oxidant for cytochromes is limited generally to the α- and β-bands. The advantages of observing spectral changes in the Soret region prompted us to test other oxidants, and ammonium persulfate proved satisfactory. It elicited spectral changes in cytochrome o solutions in the α- and β-regions that were identical with those elicited by ferriyanide and, because it is colorless, absorbancy changes in the Soret region were observable also. The absorbancy changes produced by ferriyanide or ammonium persulfate, particularly the increase in absorption at 405 nm, were evidence for the existence of some nonoxidized forms of cytochrome o, that can be oxidized by ferriyanide or ammonium persulfate, in untreated preparations. Consequently, cytochrome o treated with ammonium persulfate, rather than untreated cytochrome o, was considered here to be the completely oxidized form, although spectral changes elicited by the addition of ferriyanide or ammonium persulfate to the purest preparations of cytochrome o were usually negligible.

Cytochrome o, that was chemically reduced by sodium dithio-

Fig. 1. Absolute spectrum of cytochrome o treated with NADH. Effect of dithionite and ferriyanide on this spectrum. Final concentrations: NADH, 2.5 × 10⁴ M; sodium phosphate, 0.1 M, pH 7.5. Curve A, untreated; Curve B, 30 min after addition of NADH; Curve C, 3 min after addition of dithionite to solution of Curve B, essentially identical with spectrum obtained with dithionate alone; Curve D, 3 min after addition of ferriyanide to solution of Curve B. In this and subsequent figures, curves in the inset have been offset for easier comparison.

The spectrum of the oxygenated form was elicited readily by adding NADH to purified preparations of cytochrome a (6). Absolute spectra of this form had absorption maxima at 576, 543, and 414 nm (Fig. 1B); in difference spectra the absorption maxima were at 577, 544, and 420 nm (6). The spectrum of the oxygenated form of cytochrome o (Fig. 1B) exhibited absorption maxima at wavelengths where neither the reduced nor the oxidized form of the cytochrome have significant absorbance (Fig. 1, A and C). Spectra of “mixtures” of reduced and oxidized cytochrome o in varying proportions, obtained using two cuvettes in series, one containing reduced cytochrome and the other, oxidized cytochrome, did not resemble the spectrum of the oxygenated form of cytochrome o. These observations eliminated the possibility that the latter spectrum was due to a mixture of reduced and oxidized form of the cytochrome.

The oxygenated form of cytochrome o was quite stable and existed for hours at room temperature. Spectra of solutions of this form stored for several days at 4°C remained unchanged, but when frozen, the spectrum of the oxidized form was present on thawing. In the presence of excess NADH the oxygenated form then quickly reappeared. The oxygenated form was converted to the reduced form by the addition of excess dithionite and was converted to the oxidized form by ammonium persulfate or potassium ferriyanide (Fig. 1, C and D). When NADH was added to crude preparations of cytochrome o the absorption maxima at 577, 544, and 420 nm attained maximum intensity immediately, i.e. less than 1 min after the addition of NADH (Fig. 2B). With time, the observed absorption maxima were replaced by the absorption character of the reduced form of the cytochrome (Fig. 2C and D). The reoxidation of oxygen into the solution by gently inverting the cuvette several times or bubbling with air restored the intermediate spectrum (Fig. 2E). After about 15 min the spectrum of the reduced form again returned. This further evidence that appearance of the intermediate spectrum required the presence of oxygen.
FIG. 2. Spectral changes observed as function of time after addition of NADH to cytochrome o (46 to 65% ammonium sulfate precipitate). Difference spectra. Final concentrations: cytochrome o, $A_{410} = 0.46$; extract, 0.22 mg of protein per ml; NADH, $1.5 \times 10^{-8}$ M; sodium phosphate buffer, 0.1 M, pH 7.5. Curve A, base-line; Curves B, C, and D, 2, 25, and 45 min, respectively, after addition of NADH to experimental cuvette; Curve E, cuvette inverted 10 times to admit air.

The experiment, the results of which are given in Fig. 2, was run in the presence of a respiring cell-free extract of *Vitreoscilla* to facilitate oxygen removal and decrease the time required for the appearance of the spectrum of the reduced form of the cytochrome. These cell-free extracts could be replaced by respiring whole cells of *Vitreoscilla* or even yeast, so that their only apparent function was the removal of oxygen from the solution. Indeed, the same results were obtained without any of these additions, but the time required for the appearance of the spectrum of reduced cytochrome o at low concentrations of cytochrome o in air-saturated solutions was several hours when such additions were omitted. As a qualitative test of the theory that the appearance of the reduced form of the cytochrome was due to its formation from the oxygenated form when the solution was deoxygenated the experiment illustrated in Fig. 3 was performed. The control, in air-saturated solution, exhibited a lag of 17 min after the addition of NADH before the absorbance at 435 nm began to increase, indicating the formation of the reduced form of the cytochrome (Fig. 3c). Bubbling the cuvette 2 min with nitrogen shortened the lag to 8 min, and using an anaerobic cuvette evacuated and flushed several times with nitrogen to achieve partial anaerobiosis shortened the lag to 2.5 min (Fig. 3, b and a). The lag was also shortened by increasing the amount of respiring cell-free extract or by employing higher concentrations of cytochrome o itself. These experiments showed that the reduced form of cytochrome o appeared when the concentration of dissolved oxygen fell below a certain level.

Simultaneous monitoring of the absorbance change at 435 nm and oxygen uptake as described in “Materials and Methods” yielded an estimate of the oxygen affinity of cytochrome o. These measurements were made at several concentrations of cytochrome o with and without extract to ensure that the measured oxygen affinity of the cytochrome was independent of the rate of deoxygenation of the solution. The increase in absorbance at 435 nm, which is a measure of the reduced form of cytochrome o, began when the oxygen concentration decreased to an average value of 14 μM, and this form of the cytochrome was at half maximal concentration at approximately 6 μM oxygen (Table I).

Carbon monoxide binds cytochrome o in vivo and inhibits the respiration of microorganisms possessing this terminal oxidase (12-16). The cytochrome o purified from *Vitreoscilla*, in the reduced but not the oxidized form, formed a complex with carbon monoxide (Fig. 4). When carbon monoxide was bubbled through a solution of the oxygenated form, the absorption maxima shifted and the spectrum then resembled the spectrum of the reduced form of the cytochrome.

![Image](https://example.com/fig3.png)

**Fig. 3.** Effect of different stages of anaerobiosis on time of appearance of the reduced form of cytochrome o in the presence of NADH and cell-free extract; experimental details are given under “Materials and Methods.” Final concentrations: cytochrome o, $A_{410} = 0.75$; extract, 0.24 mg of protein per ml; NADH, $6.7 \times 10^{-8}$ M; sodium phosphate buffer, 0.1 M, pH 7.5. Curve a, anaerobic cuvette evacuated and flushed three times with nitrogen, obviously still containing residual air; Curve b, bubbled 2 min with nitrogen; Curve c, no treatment, air-saturated.

<table>
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<tr>
<th>Cytochrome o concentration ($A_{410}$ initial)</th>
<th>Extract concentration (mg protein/ml)</th>
<th>Oxygen uptake (μl O2/min/ml)</th>
<th>Ox concentration where reduced form became detectable (μM)</th>
<th>Ox concentration where reduced form was half-maximal (μM)</th>
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* Average concentration.
cytochrome o-carbon monoxide complex, indicating that the oxygenated form of cytochrome o will react with carbon monoxide (Fig. 5).

Cyanide, another important inhibitor of terminal oxidases, formed a complex with the oxidized form but not the reduced form of cytochrome o (Fig. 6A). Unlike carbon monoxide, it was unable to react with the oxygenated form, even at relatively high concentrations (Fig. 6, B and C). When cyanide was added to the oxidized form, however, it inhibited formation of the oxygenated form on the addition of NADH (Fig. 7).

Evidence was presented above for the presence of some reduced form of cytochrome in untreated solutions. When oxygen was bubbled through an untreated solution of cytochrome o, absorption peaks appeared at 577, 544, and 418 nm, and a trough appeared at 435 nm in difference spectra. These peaks, although not intense, confirmed the presence of a small amount of reduced cytochrome o in untreated preparations, which could be converted to the oxygenated form. Bubbling oxygen through solutions of cytochrome o reduced by NADH, which already contained the oxygenated form, increased the intensity of the absorption bands at 577, 544, and 420 nm in difference spectra. When nitrogen was bubbled through a solution of the oxygenated form, the Soret maximum at 420 nm shifted toward higher wavelength, and a pronounced shoulder appeared at 435 nm, which apparently represents the conversion of some oxygenated form to the reduced form. Spectral shifts observed on bubbling cytochrome o solutions with oxygen or nitrogen were more pronounced with impure preparations than with purer preparations.

The nature of the bonding in the reduced cytochrome oxidase-oxygen complex is not known. It has been postulated to be a peroxidic compound based on the spectral similarity of the oxygenated form to the complex that cytochrome oxidase forms with hydrogen peroxide (5, 17, 18), but not all workers agree with this.
interpretation (19, 20). The addition of hydrogen peroxide to cytochrome o results in a spectrum similar to the spectrum of oxygenated cytochrome o (Fig. 8).

**DISCUSSION**

Spectral studies of three forms of cytochrome o and evidence for their interconversions have been presented in the experimental section. The spectrum of cytochrome o treated with ammonium persulfate or ferricyanide was the standard for the oxidized cytochrome. Excess dithionite added to solutions of cytochrome o chemically reduces both the cytochrome and the dissolved oxygen, and under these anaerobic conditions the spectrum of the reduced form of the cytochrome is observed. The oxygenated form of cytochrome o occurs in the presence of a reducing agent, NADH, and oxygen. The oxygenated form can be chemically oxidized to the oxidized form with ammonium persulfate or converted to the reduced form by dithionite. Bubbling nitrogen through solutions of the oxygenated form converted some of this form to the reduced form, whereas bubbling carbon monoxide through these solutions readily elicited the spectrum of the reduced cytochrome-carbon monoxide complex, evidence for the relatively high affinity of reduced cytochrome for carbon monoxide (Fig. 5). The oxygenated form can be produced from the oxidized cytochrome by reduction in the presence of oxygen and from the reduced cytochrome by the addition of oxygen (Figs. 1 and 2). Direct conversion of the oxidized cytochrome to the reduced cytochrome or vice versa occurs on the addition of dithionite or ammonium persulfate, respectively. Evidence for the conversion of the oxygenated form to the oxidized form with the concomitant reduction of the bound oxygen, is the finding that purified preparations of cytochrome o exhibit oxygen uptake on the addition of NADH, which is oxidized, as shown by a decrease in absorbance at 340 nm. However, the NADH-induced oxygen uptake of these purified preparations is low (6) so that the transition from the oxygenated form to the oxidized form occurs only slowly under our experimental conditions. Since this reaction does occur, however, the conversion of the oxygenated form to the reduced form of the cytochrome (Fig. 2) probably proceeds via oxidation by the bound oxygen to form the oxidized cytochrome, which is then reduced by the NADH present. Direct loss of the bound oxygen to the oxygen-depleted solution is also conceivable. The relatively slow reduction of oxygen by cytochrome o in the presence of NADH is difficult to accommodate to the presumed role of the cytochrome as a terminal oxidase, but other data, especially the photochemical action spectra and the high affinity of the cytochrome for oxygen are difficult to explain with another postulated role for cytochrome o. The interconversions of cytochrome o are summarized schematically in Fig. 9.

A possible explanation for the stability of the oxygenated form of cytochrome o, i.e. the slow reaction of the cytochrome with the bound oxygen, is that other components are required for this oxidative step in vivo, and these components are missing in the purified preparation of cytochrome o. Thus, the addition of NADH to the less pure cytochrome o of the 45 to 65% ammonium sulfate purification stage results in immediate formation of the oxygenated form (Fig. 2), and the oxygen uptake of this fraction in the presence of NADH is much more rapid than the oxygen uptake of more purified fractions per unit of cytochrome o, although not per unit of protein (6). Whether these higher activities of the less pure fractions are due to the presence of components required for the optimum formation of oxygenated cytochrome o and its subsequent oxidation or whether they are due to the presence of alternate terminal oxidase systems in the crude preparations requires further study. It is noteworthy that cytochrome oxidase is only slowly autoxidizable in the absence of added cytochrome o (21). Orii and Okunuki (11) have postulated that cytochrome c stimulates the reaction of reduced cytochrome oxidase with oxygen by accelerating the conversion of the oxygenated form to the oxidized form. Nevertheless there is a significant difference in the stability of the oxygenated form of cytochrome oxidase and that of cytochrome o. The former reacts at a reasonable rate to form the oxidized cytochrome, even in the absence of cytochrome c, whereas the oxygenated form of cytochrome o is stable for hours at room temperature and for days at 4°.

The argument that the oxygenated form of cytochrome oxidase is a denatured form of the cytochrome has been discount ed by further studies (21). It could also be argued that cytochrome o forms a stable complex with oxygen because it has been denatured on extraction from the cell membrane. There are, however, several lines of evidence against this. (a) Cytochrome o is extracted at neutral pH without the use of detergents, and subsequent purification procedures are all performed at neutral pH (6). (b) The carbon monoxide difference spectrum of the purified cytochrome is very similar to the carbon monoxide difference spectrum observed in whole cell suspensions (6). (c) The cytochrome is enzymatically reducible with NADH. (d) The cytochrome undergoes reversible interconversions between the reduced, oxidized, and oxygenated forms.

![Graph](https://via.placeholder.com/150)

**Fig. 8.** Reaction of cytochrome o with hydrogen peroxide. Absolute spectra, 0.1 M sodium phosphate buffer, pH 7.5. Hydrogen peroxide final concentration, 2.2 x 10^-3 M. Curve A, untreated; Curve B, 3 min after addition of hydrogen peroxide.

**Fig. 9.** Schematic summary of probable interconversions of oxidized (Oz), reduced (Red), and oxygenated (Oxy) forms of cytochrome o.
Although the existence of an oxygenated form of cytochrome oxidase has now been established, its role in the mechanism of terminal oxidation is disputed. Orii and Okunuki (11) propose that it is a functional intermediate in the cytochrome oxidase reaction, being formed by the rapid reaction of oxygen with the reduced cytochrome and then undergoing a relatively slow conversion to form the oxidized cytochrome and water. However, Wharton and Gibson (22) found that the first product detectable after the reaction of oxygen with purified, reduced cytochrome oxidase was the oxidized form, and this was then converted relatively slowly to the oxygenated form. The chemical nature of the oxygenated form of cytochrome oxidase and its identity or nonidentity with the complex formed by the cytochrome with hydrogen peroxide is also in dispute. Lemberg and his co-workers (17, 18) could find no significant spectral differences between the two complexes and consider them to be the same chemical species, whereas Orii and Okunuki (19) found distinct spectral differences between the two complexes. Cytochrome o also forms a complex with hydrogen peroxide, and the spectrum of this complex, although similar to the spectrum of the oxygenated form, exhibits some differences (Fig. 8). The destructive effect of hydrogen peroxide on proteins is always a possibility in these experiments. Thus, whether or not either of the oxygenated forms is a peroxidic compound is not established at the present time. The similarity of the spectrum of the oxygenated form of cytochrome o to the spectrum of the oxygenated form of hemoglobin suggests a similarity in the chemical bond between the heme and the oxygen in these two proteins. The nature of this bonding in hemoglobin is also not understood (23).

The reaction of hydrogen peroxide with cytochrome o and the identity or nonidentity of the complex with the oxygenated form of cytochrome o are presently being investigated in more detail. We are also studying the stability of the oxygenated form, with special attention to the search for factors that will stimulate its conversion to the oxidized form with the concomitant reduction of the bound oxygen.

REFERENCES

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