Spectroscopic and Quantitative Analysis of the Oxygenated and Peroxy States of the Purified Cytochrome d Complex of Escherichia coli*

(Received for publication, August 30, 1988)

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Oxygenated and peroxy states of the cytochrome d complex of Escherichia coli have been proposed as intermediates in the reaction mechanism of this ubiquinol oxidase. In this report, several stable states of the purified enzyme were examined spectroscopically at room temperature. As purified, the cytochrome d complex exists in an oxygenated state characterized by an absorbance band at 650 nm. Removal of oxygen results in loss of absorbance at this wavelength, which is restored upon the return of oxygen. The presence of one oxygen molecule in the oxygenated state was quantified by measuring oxygen released when excess hydrogen peroxide was added to the oxygenated state. Removal of oxygen from the oxygenated state by passage of argon generates a "partially reduced" state with an absorbance peak at 628 nm, apparently due to reduced cytochrome d. Addition of equimolar hydrogen peroxide to the fully oxidized state produces the peroxy state. This peroxy state is also formed upon addition of excess hydrogen peroxide to the oxygenated state via a stable intermediate termed "peroxy intermediate."

It is likely that 1) the oxygenated state consists of one molecule of oxygen bound to reduced heme d, and 2) there are at least two stable states that have bound peroxide at room temperature, the peroxy state and a newly discovered peroxy intermediate.

The cytochrome d complex is one of two quinol oxidases of the Escherichia coli aerobic respiratory chain (1). Under growth conditions where the availability of oxygen is restricted, the predominant oxidase is the cytochrome d complex, which has a high affinity for oxygen (Km = 0.024 μM) (2). The cytochrome o complex predominates when the cells are grown with high aeration.

The cytochrome d complex has been purified to homogeneity (3, 4) and shown to contain two polypeptides by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis (3) and three distinct redox centers, cytochromes b595, b566, and d (5). This enzyme is a 1:1 complex of the two polypeptides, subunit I (88,000 Da) and subunit II (43,000 Da) (6). The genes coding for both polypeptides have been cloned previously. Each of these stable redox states of the enzyme are utilized to further characterize the oxygenated and peroxy states. Approximately one molecule of oxygen was released per oxygenated cytochrome d complex upon the addition of excess hydrogen peroxide, indicating that the oxygenated species contains one oxygen molecule bound per complex. Deoxygenation of the oxygenated species with argon revealed evidence that the complex in this form is partially reduced and that the reducing equivalents in this species are associated with the cytochrome d moiety. Hydrogen peroxide titrations of the fully oxidized cytochrome d complex revealed that the peroxy state consists of an equimolar complex of enzyme with peroxide. Hydrogen peroxide titrations of the oxygenated species formed the peroxy state and identified a stable intermediate termed "peroxy intermediate" which appears to have peroxide and oxygen bound to the enzyme simultaneously. Each of these stable redox states of the enzyme are discussed in terms of possible reaction mechanisms of the cytochrome d complex.

EXPERIMENTAL PROCEDURES

Materials—Ubiquinone-1 was obtained as a gift from Hoffman-La Roche. Tween 20, sodium cholate, ascorbate, and dithioerythritol were obtained from Sigma. Octyl glucoside was purchased from Behring Diagnostics.

Enzyme Preparations—The cytochrome d complex was purified as described previously (3). The heme d content was estimated from the
dithionite-reduced minus air-oxidized spectrum both from membranes and from solutions of the purified enzyme. It was assumed that $\Delta A = 7.4 \text{mm}^{-1} \text{cm}^{-1}$ (5) for the absorption difference between the peak and trough at approximately 628 and 607 nm, respectively.

**Spectroscopic Analysis**—A Varian-Cary 219 spectrophotometer was used to record spectra as described previously (15). Spectra were stored on a magnetic disk using an LSI-11 minicomputer (Digital Equipment Co.) and plotted on a digital plotter (Houston Instruments).

**Obtaining the Various Redox States of the Cytochrome d Complex**—The cytochrome d complex as purified is in the oxygenated state (15). Addition of dithionite to the oxygenated state yields the reduced species. Addition of ferricyanide to the reduced state generates the oxidized species. Addition of excess hydrogen peroxide (10 mM) to either the oxygenated state, oxidized state, or reduced state yields the peroxy state. Fig. 1 summarizes these various processes.

**Ferricyanide Titrations**—Hydrogen peroxide titrations were performed with both the oxygenated and oxidized states of the enzyme. An intermediate called the peroxy intermediate was generated during the hydrogen peroxide titration of the oxygenated species before formation of the peroxy state (see Fig. 1). This peroxy intermediate was produced by adding one molecule of $\text{H}_2\text{O}_2$ per mol of cytochrome d assuming two cytochrome d moieties per complex with $\Delta A = 7.4 \text{mm}^{-1} \text{cm}^{-1}$ for the 628-nm–607-nm wavelength pair in the reduced minus oxygenated difference spectrum (5). Hydrogen peroxide was added in small aliquots, and spectra were taken every 10 to 15 min until there were no more spectral changes. As the peroxy intermediate was generated, isobestic points were apparent at 802 and 707 nm. For quantifying the amount of peroxy intermediate formed, it was convenient to measure the absorbance difference at 618 nm and 580 nm because of the lack of interference by the generation of the peroxy form.

**Assay of Catalase and Peroxidase Activities**—Catalase and $o$-dianisidine peroxidase activities were assayed as described in the Worthington Enzyme Manual (Worthington Biochemical Corp., 1972) with minor modifications. For catalase activity measurements, decomposition of 27 mM $\text{H}_2\text{O}_2$ (1.8 ml volume) was followed by oxygen evolution with a YSI model 53 oxygen electrode (Yellow Springs Instrument Co.). Stock solutions of $\text{H}_2\text{O}_2$ were made from 30% $\text{H}_2\text{O}_2$ (Mallinckrodt), and concentrations were verified spectrophotometrically using a molar absorbance index of 567 nmol of cytochrome d in an anaerobic, nitrogen atmosphere with added aliquots of 66 or 88 nmol of ferricyanide from a 10.1 mM stock solution that had been deoxygenated by bubbling through the sample. The amount of reduced cytochrome d present during the titration was monitored by measuring the absorbance change at 628 versus 607 nm.

**Hydrogen Peroxide Titrations**—Hydrogen peroxide titrations were performed with both the oxygenated and oxidized states of the enzyme. Addition of excess hydrogen peroxide (10 mM) to one molecule of $\text{H}_2\text{O}_2$ per mol of cytochrome d, assuming two cytochrome d moieties per complex with $\Delta A = 7.4 \text{mm}^{-1} \text{cm}^{-1}$ for the 628-nm–607-nm wavelength pair in the reduced minus oxygenated difference spectrum (5). Hydrogen peroxide was added in small aliquots, and spectra were taken every 10 to 15 min until there were no more spectral changes. As the peroxy intermediate was generated, isobestic points were apparent at 802 and 707 nm. For quantifying the amount of peroxy intermediate formed, it was convenient to measure the absorbance difference at 618 nm and 580 nm because of the lack of interference by the generation of the peroxy form.

In making comparisons of the various species, difference remained was determined by oxygen evolution using catalase. A control was run without oxides or peroxidase to verify the amount of hydrogen peroxide added. Both assay determinations gave similar results.

**RESULTS**

**Schematic Representation of the Stable Redox States and Difference Spectra of the Purified Cytochrome d Complex**—A summary of the various redox states and difference spectra is presented in Fig. 1. Six stable redox states of the cytochrome d complex are examined in this paper: the oxygenated state, the reduced state, the oxidized state, a partially reduced state, the peroxy intermediate, and the peroxy state. Most of the difference spectra (labeled as arrows with $\Delta_1$, $\Delta_2$, $\Delta_3$, etc. in Fig. 1) in this paper are presented relative to the "oxygenated" species. The isolated enzyme is normally in this state.

**Spectra of the Oxygenated, Oxidized, and Reduced States and the Difference Spectra between Them**—Shown in Fig. 2 are the absolute spectra of the oxygenated, oxidized, and reduced states and the reduced minus oxygenated difference spectrum ($\Delta_2$ in Fig. 1) of the cytochrome d complex. Especially noteworthy is the peak at 650 nm in the oxidized spectrum which is primarily due to oxygen binding. Removal of oxygen from the oxygenated species causes a large loss in absorbance at this wavelength, as discussed below. The spectrum of the fully oxidized form also has a low absorbance at 650 nm.
spectroscopy was particularly useful. Fig. 3 points out the distinctive features (a peak at 677 nm and a large trough at 648 nm) of the oxidized minus oxygenated spectrum (spectrum \( \Delta A \)). The oxidized species was generated by adding dithionite and ferricyanide sequentially to the oxygenated form. Ferricyanide addition directly to the oxygenated species slowly (over hours) generates an identical oxidized species. Removal of Oxygen from the Oxygenated State Yields a Partially Reduced State—Removal of bound oxygen from the oxygenated state using a flow of argon generated a partially reduced state of the enzyme which could be reversibly returned to the oxygenated state by adding back molecular oxygen. Most distinctive of the “partially reduced” minus oxygenated difference spectrum (Fig. 4, dashed line) are the loss of absorbance at 650 nm and a peak at 628 nm. The peak of 628 nm is characteristic of reduced cytochrome d (5). In order to confirm that the peak at 628 nm is indeed due to the reduced cytochrome d component of the complex, aliquots of ferricyanide (10 \( \mu M \)) were added to this partially reduced state (Fig. 5). An extinction coefficient of 7.0 \( \text{mM}^{-1} \text{cm}^{-1} \) for the absorbance change using the wavelength pair of 628 nm and 607 nm was calculated from the titration data (Fig. 5). This is in good agreement with the value of 7.4 \( \text{mM}^{-1} \text{cm}^{-1} \) for \( \Delta A \) (628 nm − 607 nm, reduced-oxygenated) reported by Lorence et al. (5), based upon coulometry of cytochrome d. This result suggests that all of the reducing equivalents of the partially reduced state are associated with the cytochrome d component of the cytochrome d complex.

**Hydrogen Peroxide Titration of the Oxygenated State**—Addition of substoichiometric amounts of hydrogen peroxide to the oxygenated form of the cytochrome d complex revealed a new species. Note the isosbestic points at 662 nm and 707 nm in Figs. 6 and 7 (spectrum \( \Delta A \)). After formation of this intermediate (labeled peroxo intermediate, Fig. 1), further addition of hydrogen peroxide yielded another species (labeled as the peroxy state, Fig. 1) that is distinct from the peroxo intermediate. The difference spectrum between the peryo state and the peroxy intermediate (spectrum \( \Delta A \), Figs. 1 and 7) is virtually identical with the difference spectrum (spectrum \( \Delta A \), Figs. 1 and 3) between oxidized and oxygenated states. Each of these spectra (spectrum \( \Delta A \) and \( \Delta A \)) has a peak at 677 nm and a trough at 648 nm. These transitions are noted in Fig. 1.

The amount of hydrogen peroxide that is required to form the peryo intermediate was measured by observing the change in absorbance (618 nm − 580 nm) as aliquots of hydrogen peroxide are added. Fig. 8 shows that each cytochrome d complex titrated with approximately one hydrogen...
peroxy molecule. The wavelength pair 618 nm and 580 nm was chosen since these are the isosbestic points in the difference spectrum (spectrum \( \Delta \), Fig. 7) of the peroxy state minus peroxide state. This choice of wavelength eliminated any interference due to formation of the peroxy state in the measurement of the amount of peroxy intermediate that is formed upon addition of hydrogen peroxide.

The peroxy state minus oxygenated spectrum (spectrum \( \Delta_4 \) in Fig. 7) is useful as a diagnostic for the formation of this peroxy state. The addition of 1 mol equivalent of hydrogen peroxide to the fully oxidized state appears to yield the same peroxy state, since the spectral change upon the addition of peroxide is identical with that due to the transition from the oxygenated to the peroxy intermediate (spectra \( \Delta_3 \) and \( \Delta_3 \)). This is also demonstrated by the nearly identical hydrogen peroxide tiritations of the oxygenated and oxidized states shown in Fig. 8.

The peroxy state is also formed by the addition of hydrogen peroxide to the fully reduced state based on the observed spectroscopic changes (not shown).

**Oxygen Release upon the Addition of Hydrogen Peroxide to the Peroxy Intermediate**—The equimolar addition of hydrogen peroxide to the oxygenated cytochrome \( d \) complex forms the peroxy intermediate (Fig. 8) and does not result in any release of oxygen (Fig. 9, first \( H_2O_2 \) addition). The addition of excess hydrogen peroxide to this peroxy intermediate released 0.74 molecule of oxygen per complex in one experiment (Fig. 9) and 0.91 molecule of oxygen per complex in another experiment (data not shown). The transition from peroxy intermediate to the peroxy state, therefore, involves the release of approximately one molecule of oxygen per cytochrome \( d \) complex.

**Examination of Catalase and Peroxidase Activities of the Purified Cytochrome \( d \) Complex and of E. coli Membranes**—The catalase and peroxidase activities of the cytochrome \( d \) complex were examined for two reasons. First, the cytochrome \( d \) complex readily forms a peroxy state that is needed to catalyze such reactions. Second, similarities of the spectra of cytochrome \( d \) complexes and catalases also suggest that the cytochrome \( d \) complex may have catalase and/or peroxidase activity (5).

As shown in Table I, both the purified cytochrome \( d \) complex and the membranes from a strain (RG101) in which the cytochrome \( d \) is the only respiratory oxidase have very low catalase and \( o \)-dianisidine peroxidase specific activities, with rates below one-thousandth that of ubiquinol-1 oxidase activity. These hydroperoxidase activities are about the same as those observed with free protoporphyrin IX (Table I) and are, therefore, insignificant. Conditions under which TMPD was
chiometrically to either the oxidized or oxygenated form of the enzyme, and the spectroscopic perturbations are identical. Complexes, which are termed the proxy-state, have been observed previously. The fully oxidized form. This is consistent with the proposal of oxygen and peroxide binding to the enzyme. Peroxide may be binding to cytochrome hbas or a second cytochrome d species in the complex. The data in this paper do not distinguish between these possibilities. In addition, it is possible that the complex with peroxide involves a single oxygen atom bound to a ferryl (Fe4+) form of one of the heme groups. This appears to be the case for a peroxy-bound form of cytochrome c oxidase involving the cytochrome α3 center.

2. The peroxy-bound forms of the enzyme are relevant to the catalytic cycle.

3. It seems that the oxidase can bind simultaneously to oxygen and peroxide. Only in the presence of excess hydrogen peroxide is oxygen displaced, apparently concomitant with a one-electron oxidation of the enzyme (Δα in Fig. 1). It is noted that oxymyoglobin (Fe3+) is converted to the ferric form (Fe4+) in the presence of excess hydrogen peroxide in a complex series of reactions involving a ferryl (Fe4+) intermediate (25). Whether the chemistry in the oxymyoglobin example is related to what is observed with the cytochrome d complex is not known. It is noted that other workers have previously postulated two different oxygen binding sites involved in the catalytic cycle of the cytochrome d complex (13) and the proposition of oxygen and peroxide binding to the enzyme simultaneously is at least consistent with this model. Poole and Williams (21) have also mentioned the possibility that excess peroxide results in the displacement of oxygen from the enzyme.

The schematic in Fig. 1 is based primarily on a comparison of difference spectra. The fact that spectrum Δα = spectrum Δβ and spectrum Δα = spectrum Δδ suggests that the processes resulting in these spectral perturbations are also equivalent, as shown in Fig. 1. Experimentally, (Δα + Δα) = Δα = (Δα + Δα) where the symbols refer to the difference spectra indicated in Fig. 1. The detailed nature of each of the forms of the enzyme is not known. Peroxide may be binding to cytochrome hbas or a second cytochrome d species in the complex (15). The data in this paper do not distinguish between these possibilities. In addition, it is possible that the complex with peroxide involves a single oxygen atom bound to a ferryl (Fe4+) form of one of the heme groups. This appears to be the case for a peroxy-bound form of cytochrome c oxidase involving the cytochrome α3 center (26).

The main point of this paper is to provide an additional basis for sorting out the various forms of the cytochrome d complex which may be involved in the catalytic cycle. The important new observations are that 1) the oxygenated form is one-electron reduced, and that, after removal of molecular oxygen, the electron resides in cytochrome d; 2) there are two distinct peroxy-bound forms of the enzyme, each containing one molecule of peroxide; and 3) the enzyme can bind peroxide and oxygen simultaneously. It is premature to postulate how these forms of the enzyme relate to the function of the oxidase, but certainly there are sufficient spectroscopic tools available to further characterize these forms of the enzyme and correlate them with catalytic intermediates. This is the current challenge.

REFERENCES
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