The Purification and Characterization of the Cytochrome d Terminal Oxidase Complex of the *Escherichia coli* Aerobic Respiratory Chain*

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The aerobic respiratory chain of *Escherichia coli* is branched. In aerobically grown cells harvested in mid-exponential phase, a respiratory chain containing only b-type cytochromes is predominant. This chain contains a terminal oxidase which is a b-type cytochrome, referred to as cytochrome *a*. However, when the bacteria are grown under conditions of oxygen limitation, additional components of the respiratory chain are induced, as evidenced by the appearance of new spectroscopic species. These include a new b-type cytochrome, cytochrome *b*<sub>568</sub>, as well as cytochrome *a*<sub>1</sub> and cytochrome *d*. In this paper, a purification protocol and the initial characterization of the terminal oxidase complex containing cytochrome *d* are reported. Solubilization of the membrane is effected by Zwittergent 3–12, and purification is accomplished by chromatography with DEAE-Sepharose CL-6B and hydroxyapatite. The complex contains cytochrome *b*<sub>568</sub>, *a*<sub>1</sub>, and *d*. Analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis indicates that the complex contains only two types of polypeptides with the molecular weights estimated to be 57,000 and 43,000. The purified complex has oxidase activity in the presence of detergents, utilizing substrates including ubiquinol-1, *N*,*N*,*N*,*N*-tetramethyl-p-phenylenediamine, and 2,3,5,6-tetramethyl-p-phenylenediamine. The cytochrome *d* complex contains protoheme IX and iron, but does not contain non-heme iron or copper.

Approximately half of the cytochromes which are thought to participate in *E. coli* aerobic respiration are accounted for by this single complex. These results suggest that the *E. coli* aerobic respiratory chain is organized around a relatively small number of cytochrome-containing complexes.

The *Escherichia coli* aerobic respiratory chain has been subjected to considerable biophysical characterization, primarily using spectroscopic techniques (1–7). Fourth order finite difference analysis of the cytochrome *b* α band of reduced minus oxidized spectra suggests considerable complexity (6, 7). Application of this technique to either whole cells or membranes from cultures grown under conditions of high aeration reveals five spectroscopic components (7). One of these components must correspond to cytochrome *o*, a b-type cytochrome which serves as a terminal oxidase. When

E. coli is grown under conditions of oxygen limitation, three new cytochromes are induced (4, 7). These are cytochromes *b*<sub>568</sub>, *a*<sub>1</sub>, and *d*. Cytochrome *d* contains a chlorin (heme *d*) prosthetic group (8), binds to CO (9), and functions as part of a terminal oxidase (10) with a high affinity for oxygen (11). The role of cytochrome *a*<sub>1</sub> is unclear, and it has been reported that this component can also bind to CO (12, 13).

Recently, Reid and Ingledew (14) succeeded in solubilizing and partially purifying the cytochrome *d* terminal oxidase from *E. coli* which had been grown under anaerobic conditions in the presence of fumarate. The specific heme content of the final preparation, however, suggested that the material was not pure. The purpose of the present work is to report the purification and initial characterization of the cytochrome *d* terminal oxidase from *E. coli* grown under conditions of oxygen limitation. Previous analytical studies from this laboratory have demonstrated that under these growth conditions, a heme-containing protein complex is present in the *E. coli* inner membrane which is not observed when the cells are grown under conditions of high aeration (15). It is now shown that all three cytochromes which are induced under oxygen-limited growth conditions are components of a single complex. Utilizing a variety of artificial reductants, the purified complex exhibits oxidase activity in detergent. SDS-PAGE analysis indicates only two polypeptides.

**MATERIALS AND METHODS**

**Strain and Growth Conditions**—All work was performed using *E. coli* strain MR43L/F102 (16) (previously MR43L/F2) which was the gift of Dr. William Shipp, Brown University. The bacteria were grown at 37 °C in a 250-liter New Brunswick Model FM250 fermentor using a sparge rate of 2 cubic feet/min, and an agitation rate of 150 rpm. The medium consisted of Cohen and Rickenberg salts (17) plus 0.15% casamino acids (Difco, technical grade) and 0.5% sodium bi-lactate (Sigma), pH 7.0. The cells were harvested using a Sharples centrifuge 6 h after reaching stationary phase and were stored at −20 °C until used.

**Purification of Cytochrome d**—All steps were carried out at 4 °C. Approximately 60 g of frozen cells were suspended in 300 ml of buffer containing 200 mM Tris, 60 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (Sigma), pH 8.5, using an electric blender. Antifoam A spray (Dow Corning Corp., Midland, MI) was used to prevent foaming. The suspension was passed through a French pressure cell at 18,000 p.s.i. three times at 5 ml/min. Unbroken cells were pelleted by centrifugation for 20 min at 10,000 × *g* in a Sorvall GSA rotor. The supernatant was then centrifuged at 200,000 × *g* in a Beckman type 60 Ti rotor for 1 h. (All centrifugal forces are specified for the bottom of the tubes.) The pellet was solubilized in 50 ml of 75 mM potassium phosphate buffer, pH 6.3, containing 150 mM KCl, 5 mM EDTA, and 60 mM Zwittergent 3–12 (*N*-dodecyl-*N*,*N*,*N*-dimethyl-3-ammonio-1-propanesulfonate) detergent (Calbiochem-Behring). A TenBroeck tissue grinder was used to disperse the pellet. Following centrifugation at 200,000 × *g* for 1 h, the supernatant was loaded on DEAE-

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1. The abbreviations used are: SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TMPD, *N*,*N*,*N*,*N*-tetramethyl-p-phenylenediamine.

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E. coli Cytochrome d Terminal Oxidase

Sepharose CL-6B (Pharmacia) column (15 × 4.5 cm) was eluted at 300 ml/hr with a 1600 ml gradient running from 150 to 250 mM KCl in buffer containing 75 mM potassium phosphate, 5 mM EDTA, 6 mM Zwittergent-3-12, pH 6.3. 15-ml fractions were collected. All fractions with a ratio of A415/A280 greater than 0.6 were pooled and loaded directly on a fresh Bio-Rad DNA grade hydroxyapatite column (6 × 2.5 cm). The column was eluted stepwise at a rate of 50 ml/hr with the following buffers: 75 ml of 275 mM potassium phosphate, 25 mM potassium chloride (Sigma, uncryocryellized, purchased in acid form), pH 8.2 (Buffer A); 125 ml of 425 mM potassium phosphate, 25 mM potassium chloride, pH 8.2 (Buffer B). Fractions of 2 ml were collected and those fractions with an A415/A280 ratio greater than 0.6 were pooled. The pooled fraction was concentrated to 4 ml by ultrafiltration through a column XM-50 (Amicon) and dialyzed overnight against l liter of 10 mM Tris, 16 mM sodium chloride, pH 8.2.

**Protein Estimation**—Protein was estimated using the method of Lowry (18) modified so that the final solution contained 1% SDS. Bovine serum albumin (Cohn fraction V, Sigma) was used as a standard. Standard solutions contained the same buffer, etc., as the unknown samples. The correct protein concentration for the purified cytochrome d complex was found by multiplying the value obtained using the Lowry assay by 0.88. This factor was determined by quantitative amino acid analysis of the purified cytochrome d. The bovine serum albumin concentration was determined spectrophotometrically using 6.75 E%/mg.

**SDS-PAGE**—Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed as described by Laemmli (20) except that the samples were prepared for incubation by 15 min at room temperature in buffer containing 6% SDS. Electrophoresis reagents were from Bio-Rad. Gels were stained with Coomassie brilliant blue (R-250) in methanol:water:acetic acid 5:6:11 and destained in the same solution without the dye. A silver stain (Bio-Rad) was also used. Molecular weights were determined from a Ferguson plot (21) by measuring the dependence of the electrophoretic mobility of each protein on the percentage of acrylamide of the gel, 10, 12.5, and 15% acrylamide gels were run.

Gels were stained for heme (22) by incubating them in the dark for 1 h in 3.6 mM 3,3',5,5'-tetramethylbenzidine, 30% methanol, 70% sodium acetate (250 mM), pH 5.5. Color development was initiated by the addition of 400 μl of hydrogen peroxide (30%).

**Spectroscopy**—Spectra were recorded using either an Aminco DW2 or a Varian Cary 219 spectrophotometer. Spectra were recorded at a rate of 0.5 mm/s using a 1 mm bandwidth and a 1-s time constant. Samples were reduced with sodium dithionite (hydroxylsulfite) and oxidized using either 0.1 M potassium ferricyanide or air. CO binding to the complex was accomplished by bubbling the solutions with the gas until the maximum spectroscopic effect was obtained. Low temperature spectroscopy (77 K) was performed using the Aminco DW2 accessory designed for that purpose.

**Pyrogenesis**—The following steps (23) were carried out at 4°C in the dark. A cytochrome d solution (0.5 ml) was extracted three times with 5 ml of acetone containing 3% (v/v) 12N HCl. The extracts were pooled and neutralized with 2 ml of 3 M sodium acetate and extracted with ether which had been extracted previously with aqueous ferrous ammonium sulfate to remove peroxides. The ether extract was concentrated almost to dryness at room temperature in a rotary evaporator and then dissolved in 1 ml of pyridine and 1 ml of trifluoroacetone (Fluka, Hauppage, NY). Assays were performed in a buffer containing 100 mM potassium phosphate, 0.05% Triton X-100, pH 7.0, at 37°C.

**Kinetic Measurements**—Oxygen utilization was measured with a YSI model 52 oxygen electrode (Yellow Springs Instrument Co., Ohio). Assays were performed in a buffer containing 100 mM potassium phosphate, 0.05% Triton X-100, pH 7.0, at 37°C. The substrates used were 2,3,5,6-tetramethyl-p-phenylenediamine (Fluka, Hauppage, NY), TMPD (Sigma), and ubiquinol-1 (kind gift of Hoffmann-LaRoche). When ubiquinol-1 was the substrate, 5 mM ubiquinone-1 was included in the assay mix. The reported values are the averages of three hydrolyses on different preparations of the enzyme.

Table I summarizes the results of a representative preparation of the cytochrome d complex. High levels of cytochrome d are produced by using a strain carrying the F152 episome (16), by using lactate as a growth substrate, and by allowing the cells to go well into stationary phase under conditions of low aeration. To inhibit E. coli proteases (35), the cells are broken in a French pressure cell in the presence of EDTA and phenylmethylsulfonyl fluoride. The inclusion of L-1-to-2-hydroxamino-2-hydroxy-3-octyl-1,4-naphthoquinone (Aldrich) was used.

**RESULTS**

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to homogeneity. The elution profile of the DEAE-Sepharose C1-6B column is shown in Fig. 1. The cytochrome d elutes in two peaks. The second of the two peaks has a higher specific heme content and is pooled and loaded on a hydroxyapatite column (Fig. 2). The SDS gel of the final product, shown in Fig. 3, shows two polypeptides which stain with Coomassie brilliant blue. When a silver stain is used, some material stains which is at the dye front of 12.5% polyacrylamide gels. The nature of this material is under investigation. Attempts to further purify the cytochrome d complex were unsuccessful and included gel filtration chromatography, ammonium sulfate precipitation, sucrose density gradient centrifugation, hydrophobic chromatography, and chromatofocusing. In all cases, the two subunits remain associated. The purified complex migrates as a single protein band in polyacrylamide gel electrophoresis in the presence of Triton X-100. This band contains heme (15) and exhibits TMPD oxidase activity (15) and contains both polypeptide subunits observed in SDS-PAGE analysis. Isoelectric focusing was also performed on the purified complex in the presence of Triton X-100. A single band is again observed. This band contains heme (15) and exhibits TMPD oxidase activity (15). The pI is 5.3.

**Molecular Weight of the Subunits**—SDS-PAGE was carried out using the Laemmli system (20) with acrylamide concentrations varying from 10 to 15%. The molecular weight estimate of the smaller subunit was found to be a function of the percentage of acrylamide in the gel. For this reason, a Ferguson analysis (21) was performed in order to estimate the molecular weight of the two subunits (Fig. 4). The results of three separate determinations yield subunit molecular weight estimates of 57,000 and 43,000 with a standard deviation of approximately 5% in each case. Using 12.5% acrylamide gels, the lower molecular weight subunit migrates as an M, = 28,000 species. The Ferguson plot analysis was the same using the Weber and Osborn gel system (36) and was unchanged by the addition of 8 M urea to the buffers.

**Table I**

**Purification of cytochrome d complex.**

<table>
<thead>
<tr>
<th>Step</th>
<th>Total cytochrome b</th>
<th>Total protein</th>
<th>Specific heme content</th>
<th>Yield %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole cells (60 g)</td>
<td>9350</td>
<td>7150</td>
<td>1.3</td>
<td>100</td>
</tr>
<tr>
<td>Membranes</td>
<td>6280</td>
<td>1440</td>
<td>4.4</td>
<td>67</td>
</tr>
<tr>
<td>Zwittergent 3-12 extract</td>
<td>5210</td>
<td>1030</td>
<td>5.1</td>
<td>56</td>
</tr>
<tr>
<td>DEAE-Sepharose CL-6B pool</td>
<td>1620</td>
<td>143</td>
<td>11.3</td>
<td>17</td>
</tr>
<tr>
<td>Hydroxyapatite pool</td>
<td>617</td>
<td>31.8</td>
<td>19.4</td>
<td>7</td>
</tr>
</tbody>
</table>

*Computed using Α₅₆₀ = 10,800 M⁻¹ cm⁻¹ for the b heme in the complex. This value is obtained by extraction and measurement of protoheme IX as described in the text.

![Fig. 1. Elution profile of the DEAE-Sepharose C1-6B column.](image1)

The column volume was approximately 270 ml. Following the application of the sample (50 ml), the cytochrome was eluted using a salt gradient as described in the text. The cytochrome-containing fractions which were pooled are indicated by the bar.

![Fig. 2. Elution profile of the hydroxyapatite column.](image2)

The column volume was 30 ml. The pooled fractions from the DEAE-column (150 ml) were applied, followed by stepwise elution as described in the text. The cytochrome-containing fractions which were pooled are indicated by the bar.

![Fig. 3. SDS-polyacrylamide gel electrophoresis of the purified cytochrome d complex (10 μg).](image3)

The Laemmli system was used as described in the text. The gel was 12.5% acrylamide. The apparent molecular weights of the two subunits were determined using a Ferguson plot analysis to be 57,000 and 43,000. The values obtained from the 12.5% gel were M, = 55,000 and 28,000. Densitometric scans indicate that the two subunits account for over 90% of the Coomassie-stained material using gels with as much as 50 μg of protein.

**Spectroscopic Studies**—The optical spectra of various forms of the cytochrome d complex are shown in Figs. 5–8. The reduced minus oxidized difference spectrum (Figs. 7 and 8) clearly shows peaks diagnostic for cytochromes b (560 nm), a₁ (595 nm), and d (628 nm). The low temperature spectrum (Fig. 8) shows that the peak associated with b₁0 is not split and probably represents a single component. There is a dis-
Fig. 4. Ferguson plot analysis of the apparent molecular weights of the two subunits of the cytochrome d complex. A, the dependence of the relative mobility ($R_f$) on the percentage of acrylamide. The solid lines (1, 2, 4, 6, and 7) represent bovine serum albumin (66,000), catalase (60,000), ovalbumin (45,000), carboxypeptidase A (33,600), and chymotrypsinogen (25,000). The dashed lines (3 and 5) represent the two cytochrome d subunits. B, the slopes of the lines in A are plotted versus the molecular weight of the standard proteins. The apparent molecular weights of the two subunits of the cytochrome d complex are indicated by the arrows. The values obtained were $M_1$, 57,000 and 43,000.

Fig. 5. Absorption spectra at 25 °C of the air-oxidized form of the cytochrome d complex. The samples contained 0.14 mg/ml of protein (2.94 μM protoheme IX, 5.32 μM iron by neutron activation analysis) in 10 mM Tris, 16 mM sodium cholate, pH 7.8. Higher concentrations of Tris cause degradation of the cytochrome. Spectra shown are air-oxidized (---) and air-oxidized plus CO (— — —). Procedures are described in the text. The addition of 1 mM potassium ferricyanide did not affect the spectrum of the air-oxidized form of the cytochrome.

Fig. 6. Absorption spectra at 25 °C of dithionite-reduced forms of the cytochrome d complex. Conditions were identical with those described in the legend to Fig. 5. ---, dithionite-reduced; — — —, dithionite-reduced plus CO.

was determined using the pyridine hemochromagen technique. The spectrum of the hemochromagen (not shown) is typical of protoheme IX (23). The heme was extracted from several different preparations of the enzyme as well as with several organic solvents. The extinction coefficient of 10,800 M⁻¹ cm⁻¹ for the wavelength pair 562–580 nm was determined for the difference spectrum of cytochrome $b_{562}$. This is the average of eight extractions with a deviation of about ±10%. Neither hemes $a_1$ or $d$ were detected with the protocols utilized, although both have been extracted previously from E. coli membranes (8, 40) using essentially the same techniques.

Metal Analysis—Metal analyses were performed by neutron activation and the results are summarized in Table II. It is concluded that the only metal which is present in significant quantity is iron, which is present at 34.1 nmol/mg of protein (1.8 nmol/nmol of heme $b$). The values for iron (nanomole/mg) obtained colorimetrically (30.2 ± 6.8, three determinations) and by atomic absorption (25.5 ± 1.5, six determinations) are somewhat lower. This is not unexpected in the case of atomic absorption since the protocol followed did not include prior sample pyrolysis and metal occlusion could have resulted. However, cytochrome $c$ standards analyzed under the identical conditions gave the correct results.

Distinct shoulder near 547 nm in a region normally associated with c-type cytochromes. However, no covalently bound heme was apparent by heme staining the subunits of the complex following SDS-PAGE. Also, no heme $c$ was released by silver from the protein following extraction with acetone. Preliminary electrochemical studies suggest that this optical transition may be associated with the cytochrome $a_1$ component.

Figs. 5 and 6 show that both the dithionite-reduced and air-oxidized forms of the oxidase bind to CO. However, the fact that cytochrome $d$ can form a stable intermediate which may not be fully oxidized (38, 39) complicates the spectroscopic analysis. The spectroscopic perturbations due to CO binding (Figs. 7 and 8) are largely associated with cytochrome $d$, although interaction with cytochrome $a_1$ may also be occurring.

The extinction coefficient of the cytochrome $b$ component

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J. G. Koland, M. J. Miller, and R. B. Gennis, unpublished results.
protein. This corresponds to 1 mol of protoheme IX/53,000 g preparations. The polarity percentage (41)
ratios of approximately 18.9 nmol of heme b/mg of
the cytoplasmic membranes.

**Preliminary EPR Studies**—The EPR spectra of the
air-oxidized and dithionite-reduced forms of the enzyme were recorded using a Bruker ER200D spectrometer at 4 K at pH 8 in 10 mM Tris, 15 mM cholate. The spectrum of the
air-oxidized sample is typical of high spin ferric heme with a g
value of 6.0 and a g value of 2.0. The addition of dithionite
dependent on the detergent and phospholipids which are present in the assay mixture, but a systematic examination of these variables was not performed. Also, the product of the
reduction of oxygen has not been determined. The complex does not function with horse heart ferrocytochrome c as a substrate.

Inhibition studies of the ubiquinol-1 oxidase activity are summarized in Table V. Cyanide, azide, and 2-heptyl-4-hydroxyquinoline N-oxide were effective inhibitors, but at relatively high concentrations.

**Quantitative amino acid analysis** was performed to accu-
rately determine the protoheme IX to protein ratio. Based on
three determinations, this ratio is 18.9 nmol of heme b/mg of
protein for other moieties. The data show that there are

**Steady state kinetic parameters of the cytochrome d complex**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_m$ (mM)</th>
<th>$V_{max}$ (μmol O₂/min·mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ubiquinol-1</td>
<td>0.10</td>
<td>383</td>
</tr>
<tr>
<td>2,3,5,6-Tetramethyl-p-phenylenediamine</td>
<td>0.28</td>
<td>270</td>
</tr>
<tr>
<td>TMPD</td>
<td>0.68</td>
<td>126</td>
</tr>
</tbody>
</table>
Table V

Inhibition of ubiquinol-1 oxidase activity

Activity in the absence of inhibitors was 190 μmol of O2/min mg. Assay conditions were as described in the text with 210 μM ubiquinol-1.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Control activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
</tr>
<tr>
<td>Cyanide (1 mM)</td>
<td>10</td>
</tr>
<tr>
<td>2-Heptyl-4-hydroxyquinoline N-oxide (40 μM)</td>
<td>10</td>
</tr>
<tr>
<td>Azide (15 mM)</td>
<td>38</td>
</tr>
<tr>
<td>Antimycin A (80 μM)</td>
<td>60</td>
</tr>
<tr>
<td>2-Thienyltrifluoracetone (1 mM)</td>
<td>65</td>
</tr>
<tr>
<td>2-Hydroxy-3-oxctyl-1,4-naphthaquinone (50 μM)</td>
<td>70</td>
</tr>
<tr>
<td>Bathophenanthroline (150 μM)</td>
<td>96</td>
</tr>
<tr>
<td>Rotenone (100 μM)</td>
<td>107</td>
</tr>
</tbody>
</table>

Eliminates the EPR signal. No EPR signals corresponding to copper, non-heme iron, or semi-quinone were observed.

Discussion

Most bacteria have branched respiratory chains (42-44) and frequently possess multiple terminal oxidases. The four recognized classes of the oxidases are αα2, α1α0, δ, and d types (45). Cytochrome d terminal oxidases are found in many bacteria (43) besides E. coli, including Azotobacter vinelandii (45). An oxidase which appears similar to the one reported in this paper has been isolated from Photobacterium phosphorum (46). In the case of E. coli, it has been speculated that cytochrome d is induced under conditions of low oxygen tension due to the fact that this cytochrome has a higher affinity for oxygen than does the other major oxidase in E. coli, cytochrome b5 (11). Cytochrome o and cytochrome b558 are the predominant cytochrome components when E. coli is grown under high aeration (42), and both have been purified (47,48).

Under oxygen-limited growth conditions, E. coli produces three additional cytochrome respiratory chain components, cytochromes b566, α1, and d (4,7). The same components are also produced under other growth conditions and appear to be induced together (49, 50). Furthermore, a mutant of E. coli has been characterized in this laboratory which is lacking cytochromes d, α1, and b558 (51). This mutant appears to result from a single lesion in the E. coli genome. The main point of this work is that all three cytochrome components identified with the low aeration branch of the respiratory chain are part of a single complex. This complex possesses ubiquinol-1 oxidase activity and corresponds to the membrane component which is present in cells harvested in the stationary phase of growth which was previously identified (15) using crossed immuno-electrophoresis and isoelectric focusing.

Analysis by SDS/polyacrylamide gel electrophoresis indicates that the complex contains two kinds of subunits. The electrophoretic mobility of the low molecular weight subunit relative to the usual protein standards is a function of the percentage of acrylamide. The molecular weight which is reported is a result of a Ferguson plot analysis. This could be wrong if the Stokes radius of the SDS-protein complex of this subunit is not related to the true molecular weight in the same manner as it is the protein standards.

The spectroscopic analysis of the pure complex shows that cytochromes b566, α1, and d are all present. Cytochrome b566, which is present in the membrane and in the preparation of Reid and Ingledew (14), is not present in the purified cytochrome d complex. The shoulder observed at 547 nm in the low temperature difference spectrum (Fig. 8) is not due to a c-type cytochrome but must be assigned to another prosthetic group. Possibly, cytochrome α1 has a difference spectrum similar to high spin heme α compounds (52) or the α0 component of cytochrome c oxidase (53). This might explain the optical transition near 550 nm and also the relatively small extinction coefficient of the transition at 595 nm due to cytochrome α0.

Studies with CO indicate that the primary site of interaction is cytochrome d, although cytochrome α1 may also be involved. It has been claimed that cytochrome α1 can bind to CO (12, 13) and function as a terminal oxidase (12). It is clear from these data, however, that cytochrome α1 should not be considered as an independent entity, but is in a complex with cytochrome d. The relative amounts of cytochrome α1 and cytochrome d are approximately the same, both in the pure material and in the E. coli membrane as judged by difference spectroscopy. The optical transition at 595 nm (reduced minus oxidized spectrum) is clearly not an optical band associated with cytochrome d, as demonstrated by potentiometric titrations which reveal different midpoint potentials for these two components (4,5). Metal analysis also makes it clear that the optical transition at 595 nm is not due to copper. The elucidation of the nature of the cytochrome α1 component must await future studies.

The features in the difference spectra in the b-region due to CO binding to the dithionite-reduced form of the oxidase may be due to some denatured cytochrome b. It should be noted that if the spectrum of the purified complex is the same for the cytochrome in the membrane, then the presence of this complex will interfere with the traditional measurements of cytochrome b by CO difference spectroscopy (e.g., Ref. 54).

Clearly CO binds to the air-oxidized form of the oxidase. It is possible that even in the presence of ferricyanide the air-oxidized form of the oxidase may be partially reduced. Further studies are in progress to elucidate the various forms of this enzyme.

The heme extraction procedure yielded only protoheme IX, corresponding to cytochrome b566. Low temperature spectroscopy (Fig. 8) suggests that a single b cytochrome is present in the complex. Hemes d and a are labile and are apparently destroyed under the conditions utilized for the analysis. The extinction coefficient which was obtained is low compared to other b cytochromes (e.g., Refs. 47, 55, and 56) and could possibly be in error. It should be noted, however, that the heme extraction procedure should yield an upper limit for the extinction coefficient since inefficient extraction will yield a high value. The spectrum of the pyridine hemochromogen appears normal. Unless the chlorin breaks down upon extraction to a form which is indistinguishable from protoheme IX, it is unlikely that heme d is interfering with the heme b analysis. Breakdown of a chlorin-like prosthetic group under these conditions has been observed (57), and the possibility that this may be occurring requires further study.

The specific b-heme content determined by extraction is 1 mol/53,000 Da and an iron to heme ratio of approximately 1.8. The data indicate 3-4 mol of iron/100,000 Da. This might suggest that the ratio of hemes b566d is 2:1.1. However, other models cannot be ruled out on the basis of these data. More extensive studies are required to characterize the prosthetic groups and to define the stoichiometry.

The amino acid analysis shows the cytochrome d complex to be hydrophobic, with a polarity index of 35%, typical for membrane proteins (41). This is consistent with the presence of phospholipid in the final preparation. It is not clear whether the quinone which is present in the complex is specifically or nonspecifically bound.
The cytochrome \( d \) complex functions as an oxidase with ubiquinol-1, 2,3,5,6-tetramethyl-\( p \)-phenylenediamine, and TMPD as reductants. It appears likely that this complex serves in vivo as a ubiquinol oxidase and accepts electrons directly from ubiquinol-8 in the \( E. \ coli \) cytoplasmic membrane. Reconstitution studies to investigate this possibility are presently underway.

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