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 $o$. 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 $b_{558}$, as well as cytochrome $a_1$ 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 hydroxypapite. The complex contains cytochrome $b_{558}$, $a_1$, and $d$. Analysis by sodium dodecyl sulfate-polyacrylamide gels 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 protoporphyrin 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 difference analysis of the cytochrome $b$-$a$ 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

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* Supported in part by National Institutes of Health Grant HL 16101 and Department of Energy Grant DE-AC02-80ER10682. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Supported by a National Institutes of Health Traineeship GM-07283.

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E. coli is grown under conditions of oxygen limitation, three new cytochromes are induced (4, 7). These are cytochromes $b_{558}$, $a_1$, 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 $a_1$ 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 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 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, $N,N',N''$-tetramethyl-$p$-phenylenediamine.
Seaphorose CL-6B (Pharmacia) column (15 \times 4.5 \text{ cm}) which was eluted at 300 ml/h with a 1600 ml gradient running from 150 to 250 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 $A_{450}/A_{280}$ greater than 0.6 were pooled and loaded directly on a fresh Bio-Rad DNA grade hydroxyapatite column (6 \times 2.5 \text{ cm}). The column was eluted stepwise at a rate of 50 ml/h with the following buffers: 75 ml of 275 mM potassium phosphate, 25 mM potassium cholate (Sigma, unrecrystallized, purchased in acid form), pH 8.2 (Buffer A); 125 ml of 425 mM potassium phosphate, 25 mM potassium cholate, pH 8.2 (Buffer B). Fractions of 2 ml were collected and those fractions with an $A_{450}/A_{280}$ ratio greater than 0.8 were pooled. The pooled fraction was concentrated to 4 ml by ultrafiltration (Amicon XM-50 membrane) and dialyzed overnight against 1 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 quantitavive amino acid analysis of the purified cytochrome d. The bovine serum albumin concentration was determined spectrophotometrically using 0.76 and 1.18 mg/ml for 550 and 560 nm, respectively.

SDS-PAGE—Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed as described by Laemmli (20) except that the samples were prepared for incubation by incubation for 15 min at room temperature in buffer containing 6% SDS. Electrophoresis reagents were from Bio-Rad. Staining of SDS-PAGE gels was performed by the method of Coomassie brilliant blue R 250 in methanol:acetic acid (5:5:1) 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 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 \mu M 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 bandpass and a 1-s time constant. Samples were reduced with sodium dithionite (hydrosulfite) and oxidized using either 1 M potassium phosphate or 1 M sodium phosphate containing 0.2 M NaOH. The oxidized state was achieved by bubbling the solutions with the gas until the maximum spectrophotometric effect was obtained. Low temperature spectroscopy (77 K) was performed using the Aminco DW2 accessory designed for that purpose.

**Pyrogens**—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) 12-diethylnitrosamine and 3% (v/v) 12-diethylnitrosamine. 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 0.2 M NaOH. A reduced minus oxidized spectrum was taken immediately. Excess dithionite was avoided in reducing the material in the sample cuvette. The quantity of protoporphyrin IX was determined from the absorbance using the wavelength pair 558-542 nm and an extinction coefficient of 20,700 M⁻¹ cm⁻¹ (23).

**Metal Analysis**—Samples of cytochrome d prepared for metal analysis by dialysis for 12 h against 1000 ml of 500 mM NaCl, 100 mM EDTA, 10 mM Tris, 15 mM sodium cholate, pH 8.3. This was followed by dialysis against two changes of 1000 ml of 10 mM Tris, pH 8.5, containing 5 mM EDTA and 15 mM sodium cholate for 12 h each. An aliquot was used to determine the reduced minus oxidized spectrum and the oxidase activity. All analyses were corrected for the trace metals contained in the final dialysis buffer. Atomic absorption analysis was performed with an Instrumentation Laboratories model 109 dual beam atomic absorption spectrometer. Known standards of horse heart cytochrome c (Sigma) were prepared in an identical manner as the unknown samples and used in controls. Environmental Protection Agency-certified trace metal standards were also used.

For neutron activation analysis, a 500-\mu l sample was pipetted into a pre-cleaned polyethylene vial and heat-sealed. Iron comparison standards were prepared by pipetting 10 and 20 \mu l of a 1000 mg/ml ferrocyanide solution and 500 \mu l of 15 Molar oxalic acid buffer into other such vials. An analytical blank of 500 \mu l of deionized water was also prepared. These samples and standards were irradiated for 8 h at a flux of 4 \times 10¹⁰ neutrons/cm² s in the rotating sample holder of the Illinois Advanced TRIGA Reactor Facility. After 2 weeks, the samples were counted 24 h each using a 14.3% efficient Ge(Li) gamma-ray spectrometer with an energy resolution of 1.80 keV at 1332 keV. The spectral data were accumulated in a computer-based multichannel analyzer that intercompares the sample and standard spectra and calculates the elemental concentrations. Blank values were found to be of the order of the calculated uncertainties in the concentrations. Molybdenum (24), tungsten (25), non-heme iron (26) were determined colorimetrically using standard procedures. Other Analyses—Total flavin (27), acid-labile sulfite (28), ubiquinone (29), menaquinone (29), phosphorus (30), carbohydrate (31), and heme c (32) were determined using published procedures.

**Amino Acid Analysis**—Approximately 175 \mu g of protein were placed in a hydrolysis tube with 90 mmol of norleucine (Pierce Chemical Co.) to serve as an internal standard and 100 \mu l of 10% SDS to prevent precipitation of the cholate upon addition of acid. The solution was made 6 N in HCl. Lipids were extracted by shaking the sample with two 4-ml volumes of diethyl ether. The residual ether was evaporated to dryness and the resin was washed with 3-ml portions of deionized water. After hydrolysis at 110°C for either 24, 48, or 72 h, samples were removed from the tubes and dried down at 80°C under nitrogen. The residue was dissolved in 250 \mu l of 0.2 M sodium citrate, pH 2.2. 100-\mu l aliquots were analyzed on a Beckman model 190 amino acid analyzer. The samples were derivatized to reduce the measured quantities of the amino acids for losses during transfers. The reported values are the averages of three hydrolyses on different preparations of the enzyme.

**Kinetic Measurements**—Oxygen utilization was measured with a YSI model 52 oxygen electrode (Yellow Springs Instrument Co., Ohio). Initial rates of the reaction were determined with the dependence on the concentration of the oxidase and the pre-equilibrated with the inhibitors. The 10 mM Tris, 16 mM sodium cholate, pH 8.2. The substrates used were 2,3,5,6-tetramethyl-p-phenylenediamine (Fluka, Hauppauge, NY), TMPD (Sigma), and ubiquinol-1 (kind gift of Hoffmann-LaRoche). When ubiquinol-1 was the substrate, 5 mM EDTA and 1 mg/ml of asolectin (Associated Concentrates) were included in the assay mix. 2,3,5,6-Tetramethyl-p-phenylenediamine was recrystallized from ethanol. Ascorbate (4 mM) was used to maintain the 2,3,5,6-tetramethyl-p-phenylenediamine and TMPD in the reduced state, and 4 mM dithioerythritol (Sigma) was used to reduce the ubiquinone 1. The turnover of the enzyme was calculated assuming the concentration of 1,0.5 in air-saturated buffer at 37°C is 25 mM. Cornish-Bowden plots were used to determine the kinetic parameters. Inhibition studies were performed with ubiquinol-1 as substrate. The cytochrome samples were pre-equilibrated with the inhibitors for 10 min prior to adding the ubiquinol-1. The oxygen consumption was measured with the presence of ubiquinol-1. The slope of the plot of the reaction velocity was used to compute the extent of inhibition. Inhibitors used included rotenone (Sigma), baphothepanol (Sigma), 2-thienyl trifluoroacetone (Fluka), antimycin A (Sigma), 2-heptyl-4-hydroxyquinoline N-oxide (Sigma), and 2-hydroxy-3-octyl-1,4-naphthoquinone (Aldrich).

**Results**

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 F1522 epoxide (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-l-threonine, L-1-threonine, L-1-phenylalanine, and L-1-phenylalanine did not provide additional protection against proteases. Sonication was used to disrupt the cells in several preparations, but the specific heme content of the final product was lower.

Efficiency as well as selectivity in the solubilization of cytochrome d was optimal using Zwittergent 3-12. Two column chromatography steps are required to purify the complex.
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 exhibits TMPD oxidase activity (15) and contains both polypeptide subunits observed in SDS-PAGE analysis. Isoelectric focussing 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.

The higher molecular weight subunit runs as a diffuse band in the Laemmli gel system, similar to what has been observed with some bacterial oxidases of the aa3 type (37). The high molecular weight subunit of the complex aggregates to a form which will not enter the polycrylamide gel if the sample is heated prior to loading onto the gel. Aggregated material which will not enter the gel is observed in some preparations.

**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), a (585 nm), and d (628 nm). The low temperature spectrum (Fig. 8) shows that the peak associated with b for 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 (Rf) 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 (33,000), 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, 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 (2.9 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.

A 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 a₁ 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 a₁ may also be occurring.

The extinction coefficient of the cytochrome b component was determined using the pyridine hemochromogen technique. The spectrum of the hemochromogen (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 b₃ₙ₅. This is the average of eight extractions with a deviation of about ±10%. Neither hemes a₁ 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.
protein. This corresponds to 1 mol of protoheme IX/53,000 g preparations. The polarity percentage (41) for the complex is shown in Table III. The values represent the average of determinations on three different preparations. The polarity percentage (41) for the complex is 35%.

Table II also shows the results of analysis of the purified cytochrome for other moieties. The data show that there are no significant amounts of acid-labile sulfide, non-heme iron, or flavin in the cytochrome d complex. The complex does, however, contain variable quantities of ubiquinone and menaquinone as well as a substantial amount of phospholipid. The ratio of quinone to phosphate is slightly greater than for the cytoplasmic membranes.

**Amino Acid Composition**—The amino acid composition of the cytochrome d complex is shown in Table III. The values represent the average of determinations on three different preparations. The polarity percentage (41) for the complex is 35%.

Quantitative amino acid analysis was performed to accurately determine the protoheme IX to protein ratio. Based on three determinations, this ratio is 18.9 nmol of heme b/mg of protein. This corresponds to 1 mol of protoheme IX/53,000 g of protein.

**Kinetics**—Steady state kinetics studies were performed using ubiquinol-1, 2,3,5,6-tetramethyl-p-phenylenediamine, and TMPD as substrates. The data (Table IV) show that the cytochrome d complex is an active oxidase. The activity is 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.

**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.

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**Table II**

Nonprotein components of the cytochrome d complex

When appropriate, the number of independent experiments is given in parentheses, and the deviation is also given. Other metals were shown to be present at less than 8% of the value measured for iron. These included Mg, Ca, Cr, Mn, Co, Ni, Zn, Se, Mo, and W. Also, no evidence was found for flavin (<0.01/heme b), heme c (<0.05/heme b), and carbohydrate (<1% of protein mass) in the cytochrome d complex.

<table>
<thead>
<tr>
<th>Component</th>
<th>nmol/mg protein</th>
<th>nmoles/heme b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iron</td>
<td>34.15 ± 0.94</td>
<td>(2)</td>
</tr>
<tr>
<td>Copper</td>
<td>0.43</td>
<td></td>
</tr>
<tr>
<td>Ubiquinone</td>
<td>4.72 ± 1.13</td>
<td>(3)</td>
</tr>
<tr>
<td>Menaquinone</td>
<td>4.72 ± 1.89</td>
<td>(3)</td>
</tr>
<tr>
<td>Acid-labile sulfide</td>
<td>&lt;0.94 (3)</td>
<td></td>
</tr>
<tr>
<td>Non-heme iron</td>
<td>0.94</td>
<td></td>
</tr>
<tr>
<td>Phosphate</td>
<td>556 ± 75</td>
<td>(5)</td>
</tr>
</tbody>
</table>

* Menaquinone and demethylmenaquinone were not distinguished.
* No non-heme iron was detectable by EPR studies on the air-oxidized or dithionite-reduced oxidase. The value given is from a colorimetric analysis (25).

**Table III**

Amino acid composition of the cytochrome d complex

Results are the average of three independent determinations.

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>mole %</th>
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<tbody>
<tr>
<td>Asx</td>
<td>8.23 ± 1.35</td>
</tr>
<tr>
<td>Thr</td>
<td>6.24 ± 0.47</td>
</tr>
<tr>
<td>Ser</td>
<td>4.80 ± 0.37</td>
</tr>
<tr>
<td>Glx</td>
<td>7.30 ± 0.65</td>
</tr>
<tr>
<td>Pro</td>
<td>4.08 ± 0.51</td>
</tr>
<tr>
<td>Gly</td>
<td>9.28 ± 0.91</td>
</tr>
<tr>
<td>Ala</td>
<td>11.50 ± 0.92</td>
</tr>
<tr>
<td>Val</td>
<td>8.78 ± 0.59</td>
</tr>
<tr>
<td>Met</td>
<td>3.45 ± 0.85</td>
</tr>
<tr>
<td>Ile</td>
<td>7.12 ± 0.30</td>
</tr>
<tr>
<td>Leu</td>
<td>12.77 ± 0.80</td>
</tr>
<tr>
<td>Tyr</td>
<td>3.44 ± 0.20</td>
</tr>
<tr>
<td>Phe</td>
<td>6.92 ± 0.34</td>
</tr>
<tr>
<td>His</td>
<td>1.49 ± 0.07</td>
</tr>
<tr>
<td>Lys</td>
<td>4.06 ± 0.43</td>
</tr>
<tr>
<td>Arg</td>
<td>1.44 ± 2.49</td>
</tr>
</tbody>
</table>

**Table IV**

Steady state kinetic parameters of the cytochrome d complex

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Assay conditions are described in the text.</th>
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<tbody>
<tr>
<td></td>
<td>$K_m$</td>
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<tr>
<td>Ubiquinol-1</td>
<td>0.10</td>
</tr>
<tr>
<td>2,3,5,6-Tetramethyl-p-phénylenediamine</td>
<td>0.28</td>
</tr>
<tr>
<td>TMPD</td>
<td>0.68</td>
</tr>
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</table>
Table V

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

E. coli Cytochrome d Terminal Oxidase

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.

DISCUSSION

Most bacteria have branched respiratory chains (42-44) and frequently possess multiple terminal oxidases. The four recognized classes of the oxidases are α, β, ε, and type (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 phosphorium (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 o (11). Cytochrome o and cytochrome b₅₆₇ 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 b₅₆₉, a₁, 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, including Azotobacter vinelandii (45), has been characterized in this laboratory which is lacking cytochromes d, a₁, and b₅₆₇ (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 immunelectrophoresis 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 for the protein standards.

The spectroscopic analysis of the pure complex shows that cytochromes b₅₆₉, a₁, and d are all present. Cytochrome b₅₆₉, 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 a₁ has a difference spectrum similar to high spin heme a compounds (52) or the a₁ 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 a₁.

Studies with CO indicate that the primary site of interaction is cytochrome d, although cytochrome a₁ may also be involved. It has been claimed that cytochrome a₁ can bind to CO (12, 13) and function as a terminal oxidase (12). It is clear from these data, however, that cytochrome a₁ should not be considered as an independent entity, but is in a complex with cytochrome d. The relative amounts of cytochrome a₁ 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 eludication of the nature of the cytochrome a₁ 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 o 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 protoporphyrin IX, corresponding to cytochrome b₅₆₉. 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 some 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 protoporphyrin 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 b₅₆₉ to a 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.

Acknowledgments—We would like to thank Robert Kranz for performing the isoelectric focusing experiments. We would also like to thank Dr. Philip Hopke of the Institute for Environmental Studies for assistance in performing the neutron activation analysis and Loretta Skowron and Barbara Warner of the Illinois State Water Survey for atomic absorption measurements. We thank Heather Mangian for operating the amino acid analyzer. We are also grateful to Neil Green, Robert Lorence, John Koland, Carolyn Deal, Ann Tully, and Amihud Morochov for assistance and helpful discussions. We also thank Michael Hendrich for assistance in EPR measurements.

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