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Coulometric and Spectroscopic Analysis of the Purified Cytochrome *d* Complex of *Escherichia coli*: Evidence for the Identification of "Cytochrome a_1" as Cytochrome b_{595}†

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ABSTRACT: Coulometric and spectroscopic analyses were performed on the three cytochrome components (cytochrome *d*, cytochrome b_{558}, and the cytochrome previously described as cytochrome a_1) of the purified cytochrome *d* complex, a terminal oxidase of the *Escherichia coli* aerobic respiratory chain. On the basis of heme extraction, spectroscopic, and coulometric data, the "cytochrome a_1" component was identified as a *b*-type cytochrome: cytochrome b_{595}. The pyridine hemochromogen technique revealed the presence of two molecules of protoheme IX per cytochrome *d* complex. This quantity of protoheme IX fully accounted for the sum of the cytochrome b_{558} and cytochrome b_{595} components as determined coulometrically. The renaming of cytochrome a_1 as cytochrome b_{595} was further indicated (1) by the lack of any heme *a* in the complex and (2) by its resolved reduced-minus-oxidized spectrum. The latter was found to be similar to that of cytochrome *c* peroxidase, which contains protoheme IX. Coulometric titrations and carbon monoxide binding titrations revealed that there are two molecules of cytochrome *d* per complex. A convenient measurement of the amount of cytochrome b_{558} was found to be the β-band at 531 nm since cytochrome b_{558} was observed to be the only component of the cytochrome *d* complex with a peak at this wavelength. By use of this method and the extinction coefficient for the purified cytochrome b_{558}, it was estimated that there is one molecule of cytochrome b_{595} and one of cytochrome b_{558} per cytochrome complex.

The branched aerobic respiratory chain of *Escherichia coli* uses two distinct terminal oxidases, the cytochrome *d* complex and the cytochrome *o* complex (Haddock & Jones, 1977; Bragg, 1979; Ingledew & Poole, 1984). Both terminal oxidases have been purified to homogeneity (Matsushita et al., 1983; Miller & Gennis, 1983; Kita et al., 1984a,b). The cytochrome *d* complex contains two polypeptides, subunits I and II, and three spectroscopically defined cytochromes, cytochromes b_{558} and *d* and the cytochrome previously described as cytochrome a_1. No prosthetic groups are present other than iron-containing hemes (Miller & Gennis, 1983). Localized mutagenesis (Green et al., 1984a) has been used to produce two classes of mutants in the cytochrome *d* complex, *cydA* and *cydB*. The *cydA* mutant lacks all three spectroscopically detectable cytochromes, and subunits I and II are not detectable immunologically. Strains manifesting the *cydB* phenotype possess only subunit I and the cytochrome b_{558} component (Green et al., 1984a).

The spectroscopic assignments of the cytochromes in the cytochrome *d* complex have been based on the absorbance maximum of the α-band of each cytochrome (reduced form). The band at 595 nm at room temperature has been attributed to "cytochrome a_1" solely by analogy with the absorbance spectra of the cytochromes a_1 of other bacteria (Poole, 1983; Castor & Chance, 1959; Cypionka & Meyer, 1983; Tanaka et al., 1983). In a potentiometric study of the purified cytochrome *d* complex, a reiterative matrix inversion (RMI)[1] method was used to generate the reduced-minus-oxidized spectrum of each electrochemically active species from the set of spectra of the complex at different oxidation/reduction potentials (Koland et al., 1984). The difference spectrum of the cytochrome a_1 component indicated an α-band at 595 nm, a strong β-band near 560 nm, and a trough near 645 nm. This spectrum is shown here to be similar to that of cytochrome *c* peroxidase which contains protoheme IX. Since no heme *a* is found in the purified complex and since extracted protoheme IX is sufficient to account not only for cytochrome b_{558} but for the cytochrome a_1 component as well, it is indicated that the prosthetic group of cytochrome a_1 is protoheme IX. Consequently, it is proposed to rename this component of the complex as cytochrome b_{595}. Coulometric titrations reveal that there are two molecules of cytochrome *d* per complex. Use of the extinction coefficient for the purified cytochrome b_{558} (Green et al., 1986) allows for the estimate that there is one

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[1] Abbreviations: RMI, reiterative matrix inversion; oxid, oxidized; oxyg, oxygenated; red, reduced.

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molecule of cytochrome b_{595} and one of cytochrome b_{558} per complex.

Materials and Methods

Spectroscopically Monitored Potentiometric Titrations. These were performed as previously described (Koland et al., 1984).

Spectral Resolution of the Components Resolved by Potentiometric Titration. Both the RMI method (Koland et al., 1984) and a simple subtraction method were used to extract the reduced-minus-oxidized spectra of each cytochrome component from a set of spectra of the cytochrome *d* complex at different solution potentials. Using as detergent 25 mM sodium cholate at pH 8.0, instead of 50 mM octyl glucoside at pH 7.0 in the titration solution, resulted in a large decrease in the midpoint potential of cytochrome b_{558} and an increased separation of the midpoint potentials of the three cytochrome components: cytochrome b_{558}, E_m = 0 mV; cytochrome b_{595}, E_m = +74 mV; cytochrome *d*, E_m = +183 mV (Lorence et al., 1984b). This separation of the midpoint potentials facilitated the resolution of the spectra of the cytochrome b_{558} and cytochrome *d* components. At low potentials only the cytochrome b_{558} component titrated, and the spectrum of cytochrome b_{558} was simply obtained. At high potentials only the cytochrome *d* component titrated. The spectrum of the cytochrome *d* component was found by taking the difference in spectra of the sample at two high potentials. The difference in spectra at two intermediate potentials (+78 and +40 mV) yielded largely the cytochrome b_{595} component spectrum. The cytochrome b_{558} interference was eliminated by subtracting the fraction of the cytochrome b_{558} component spectrum that eliminated the β-band of cytochrome b_{558} at 531 nm. To determine the relative amounts of the three spectra, their composite spectrum was optimized to fit the authentic reduced-minus-oxidized spectrum of the cytochrome *d* complex by using a least-squares analysis.

Coulometric Titrations. In the coulometric analysis of the oxidase complex, benzyl viologen and ferrocene served as mediator–titrants. The use of mediator–titrants in the coulometric analysis of enzymic systems is well documented (Heineman et al., 1972; Hawkridge & Kuwana, 1973), and procedures similar to those previously employed were adopted. Preparations for coulometric titrations proceeded exactly as described for potentiometric titrations (Koland et al., 1984), except that all mediators other than benzyl viologen (50 μM, E_m = −350 mV) and ferrocene (50 μM, E_m = +420 mV) were omitted. After deoxygenation of the cell, the cell was poised at −100 mV. Under computer control, the potentiostat delivered fixed amounts of charge at a working electrode potential of +400 mV. The absorbance of the system at a single wavelength was monitored as oxidizing equivalents were injected into the cell by the potentiostat. Thus, a profile of absorbance vs. oxidizing equivalents introduced could be obtained. The extinction coefficient of each component was directly obtained from the slope of this profile and knowledge of the sample volume (2.5–3 mL). The absolute quantity of the component could be estimated by the span of this profile (Figure 1). This protocol was effective when single components were analyzed or when the midpoint potentials of the electrochemically active species of the system were widely separated.

Before each coulometric titration of the purified cytochrome *d* complex, a test titration of horse heart cytochrome *c* (Sigma) was done. The electrochemical cell was filled with electrolyte buffer (0.1 M potassium phosphate, pH 7.0 or 8.0, 0.2 M KCl) and 42 nmol of cytochrome *c*. Octyl glucoside (50 mM) (at pH 7.0) or sodium cholate (25 mM) (at pH 8.0) was used as detergent. The absorbance at 550 nm was recorded as each 10 nequiv of charge was injected by the working electrode at intervals of 20 s. Titrations of cytochrome *d* (25 nequiv) were performed in a similar manner with each of the two detergents (octyl glucoside or cholate). Observations of absorbance as a function of injected charge were made at 628 nm for cytochrome *d* and at 561.5 nm for the sum of the cytochrome b_{558} and cytochrome b_{595} components (Figure 1B).

Carbon Monoxide Binding Titrations. Saturated solutions of carbon monoxide were prepared by equilibration of water with 1 atm of CO at 20 °C. The concentration was found to be within 5% of the literature value of 1 μM (Anderson & Antonini, 1968) by a standardization with human hemoglobin (Sigma). The value of 4.2 mM^{-1} cm^{-1} was used for the change in extinction coefficient at 538 nm occurring with CO binding to hemoglobin or myoglobin (Anderson & Antonini, 1968). Six milliliters of dithionite-reduced sample (10 μM hemoglobin or cytochrome *d*) was placed in a rubber-stoppered air-tight cuvette. The oxygen was then removed by passing argon through the system. Before each 10-nmol addition of CO from the CO-saturated solution, the spectrum of the sample was recorded in the range 500–700 nm. Titration profiles of absorbance changes (A_{538nm} for hemoglobin and $\Delta A_{647-622nm}$ for cytochrome *d*) vs. amount of CO added were obtained.

Measuring the Height of the β-Band at 531 nm of Cytochrome b_{558}. The height of the β-band of cytochrome b_{558} was measured as the absorbance differences at 531 nm between this reduced-minus-oxidized peak and a "base-line" curve at 531 nm drawn as a quadratic that fits the absorbances at 500 and 550 nm by using the absorbance at 500 nm as the origin. This procedure eliminates any interference due to cytochrome b_{595} whose spectrum is in the form of a quadratic curve in this region.

Difference Spectroscopy. Reduced-minus-oxidized and reduced-minus-oxygenated difference spectroscopy were performed as previously described (Koland et al., 1984). The former spectrum was obtained either anaerobically by oxidizing the dithionite-reduced cytochrome *d* complex with ferricyanide or electrodically during the coulometric titrations. The reduced-minus-oxygenated spectrum was obtained by subtracting the spectrum of the complex as isolated (i.e., oxygenated), in the presence of oxygen and ferricyanide, from the dithionite-reduced spectrum. The oxygenated form of the complex is generated by adding oxygen to the fully reduced form of the enzyme. The subsequent addition of ferricyanide to this form has no effect. Because of differences in the cytochrome *d* α-region (600–700 nm), one must specify whether the reduced-minus-oxidized or reduced-minus-oxygenated spectrum is used for quantifying the amount of cytochrome *d*. In contrast, no differences are observed in the cytochrome *b* α-region (500–590 nm) for the reduced-minus-oxidized vs. the reduced-minus-oxygenated spectrum.

Purification of the Cytochrome d Complex. The method of Miller & Gennis (1983) was used to purify the cytochrome *d* complex both from a strain with ubiquinone, MR43L/F152 (Shipp et al., 1972), and from one without ubiquinone. The latter was obtained by transforming the ubiquinone-lacking strain AN386 (Wallace & Young, 1977) with the plasmid pNG2, which is used to overproduce the cytochrome *d* complex (Green et al., 1984b). This allowed for overproduction and easy purification of the ubiquinone-lacking cytochrome *d* complex.

Other Methods. Pyridine hemochromogen analysis and protein assays have been previously described (Miller &

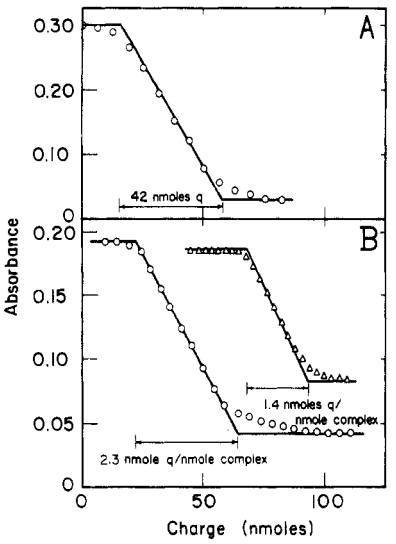

FIGURE 1: Absorbance vs. charge profiles for the coulometric titrations of (A) cytochrome *c* and (B) the cytochrome *d* complex. In (A), the absorbance was measured at 550 nm, the wavelength of the α-peak in the reduced spectrum of cytochrome *c*. In (B), the levels of reduction of the *b*-type cytochromes and cytochrome *d* were measured at 561.5 nm (○) and at 628 nm (△), respectively. These measurements were taken from oxidative titrations. The sample volume was 3.0 mL in (A) and 2.5 mL in (B).

Gennis, 1983). Purification of cytochrome b_{558} was done according to Green et al. (1986). Estimation of ubiquinone 8 was done according to Wallace & Young (1977). Purified cytochrome *c* peroxidase was a gift from Dr. James Erman of Northern Illinois University.

RESULTS

Coulometric Analysis of Cytochrome c and the Cytochrome d Complex. An attempt to quantify each cytochrome component in the functional cytochrome *d* complex was made by using spectroscopically monitored coulometry. To test the electrochemical cell, a coulometric titration of cytochrome *c* was first performed. A plot of the absorbance at 550 nm as a function of oxidizing equivalents introduced is shown in Figure 1A. One artifact encountered during the analysis of the coulometric data was the deviation from linearity observed at the beginning of the titration. The lag in absorbance change has been observed in other coulometric systems and has been attributed to the initial charging of the working electrode (Hawkridge & Kuwana, 1973).

In the course of these tests, it was discovered that reductive titrations were less quantitative (data not shown). In contrast, oxidative titrations gave clear steplike coulometric titration data (Figure 1) and were used exclusively in this report.

From the slope of the linear portion of the titration profile (Figure 1A), the extinction coefficient at 550 nm of cytochrome *c* was estimated to be 20.6 mM^{-1} cm^{-1}. This value compares to within 4% of other reported values (Massey, 1959; Van Gelder & Slater, 1962). Apparently the observed charging artifact was limited to the initial additions of oxidizing equivalents.

In the coulometric titrations of the purified cytochrome *d* complex using either detergent (cholate or octyl glucoside), it was observed that the titration could be performed more accurately if the absorbance was monitored at a single wavelength rather than having the complete spectrum recorded after addition of aliquots of oxidizing equivalents. Since cytochrome b_{558} and cytochrome b_{595} have midpoint potentials relatively close to each other in 50 mM octyl glucoside [+83 and +139 mV, respectively (Lorence et al., 1984b)], these components were not clearly resolved. Cytochrome *d* [with a midpoint potential of +256 mV under these conditions (Lorence et al., 1984b)] titrated separately and therefore was the easiest component to quantify. The absorbance at 561.5 nm reflected the reduction of both cytochrome b_{558} and cytochrome b_{595}. The absorbance at 628 nm reflected the reduction of cytochrome *d* exclusively. The absorbances at 561.5 and 628 nm are shown as a function of injected oxidizing equivalents (Figure 1B). The average of seven titrations indicates a combined quantity of 2.3 ± 0.3 mol of cytochromes b_{558} and b_{595} per mole of complex and 1.4 ± 0.3 mol of cytochrome *d* per mole of complex. These values assume a molecular weight of 100 000 for the cytochrome *d* complex, based on subunit molecular weights obtained from DNA sequence data (Gennis et al., unpublished results) and the assumption of one copy of each subunit per complex.

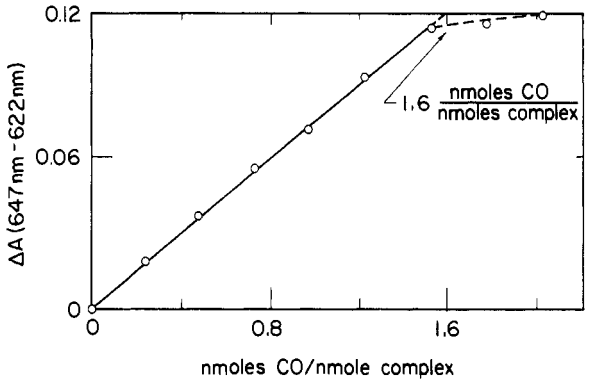

FIGURE 2: Carbon monoxide binding titration of the reduced cytochrome *d* complex. The absorbance change in the region of the spectrum where the reduced cytochrome *d* α-peak predominates was monitored by the difference at 647 and 622 nm which are the peak and trough, respectively, in the reduced plus CO-minus-reduced difference spectrum (Figure 3). This spectral change is plotted vs. the amount of CO added (in 10-nmol aliquots) to 81 nmol of cytochrome *d* in 8.0 mL. This value is obtained by using $\Delta\epsilon_{628-607,\text{red-oxyg}}$ = 7.4 mM^{-1} cm^{-1}. The amount of complex is obtained by protein measurements and by assuming a molecular weight of 100 000 for the complex. Details of the titration are described under Materials and Methods.

One possible error in these quantitative estimates of the cytochromes may arise due to the presence of ubiquinone and menaquinone in significant amounts [0.6 molecule of each per complex (Miller & Gennis, 1983)]. Estimates for the midpoint potential of ubiquinone at pH 7.0 range from 65 to 112 mV (Urban & Klingenberg, 1969). The midpoint potential of menaquinone is reported to be −57 mV at pH 7.0 (Wagner et al., 1974). Thus, although menaquinone can be apparently ignored when quantifying the cytochromes in the cytochrome *d* complex, any errors due to the ubiquinone must be addressed. Attempts to extract quinones from the complex always resulted in the denaturation of the cytochrome. Coulometric analysis of the cytochrome *d* complex purified from a ubiquinone-lacking strain yielded similar values (2.1 ± 0.3 molecules of $b_{558} + b_{595}$; 1.5 ± 0.2 molecules of cytochrome *d* per complex). Significant interference due to ubiquinone could be ruled out on this basis.

Carbon Monoxide Binding Titrations. The amount of cytochrome *d* in the complex was also determined by carbon monoxide binding titrations of the reduced cytochrome *d* oxidase complex. From the average of three trials, it is found that 1.6 ± 0.1 molecules of CO was bound per molecule of complex. This value was estimated from the linear portion of the titration curve (Figure 2). It presumably reflects the amount of cytochrome *d* in the complex since stoichiometric

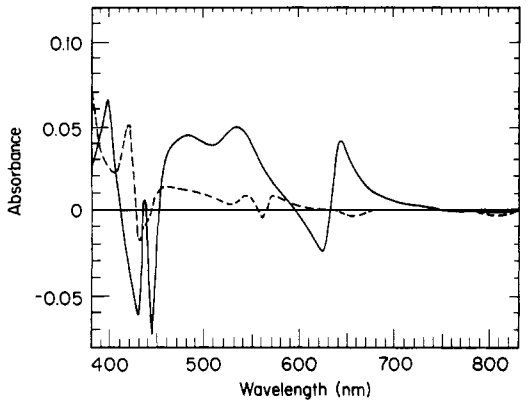

FIGURE 3: Reduced plus carbon monoxide-minus-reduced difference spectra of the purified cytochrome *d* complex. To illustrate the spectral perturbations due to carbon monoxide at low CO concentrations, the spectrum of a dithionite-reduced sample (5 μM cytochrome *d*) without CO was subtracted from a dithionite-reduced spectrum with 100 μM CO present to yield a difference spectrum (—). To illustrate spectral perturbations caused by CO at higher concentrations, the spectrum of a dithionite-reduced sample with 100 μM CO present was subtracted from the spectrum of the reduced sample saturated with CO to yield a difference spectrum (- - -).

carbon monoxide binding (i.e., at CO concentrations equal to that of cytochrome *d*) to the complex causes spectral perturbations (a peak at 642 nm and a trough at 622 nm) (Figure 3) that are largely in the region of the reduced cytochrome *d* peak at 628 nm. Both high-spin and low-spin protohemes IX are predicted to show a trough near 560 nm and a peak near 420 nm in the reduced plus CO-minus-reduced spectrum (Wood, 1984). These characteristics are lacking when a stoichiometric amount of CO is used, indicating that possible stoichiometric CO binding to cytochrome b_{558} and b_{595} is minimal.

In earlier reports (Miller & Gennis, 1983; Kita et al., 1984b) spectral perturbations due to CO were found to include a trough at 560 nm in the reduced plus CO-minus-reduced spectrum of the purified cytochrome *d* complex. Observations of this trough had indicated that CO was binding to either cytochrome b_{558} or b_{595}. However, this apparent discrepancy can be explained by our observation that the trough at 560 nm was only observed when saturating levels of CO (>100 μM) were used (Figure 3). Probably this small trough at 560 nm, which is lacking in the spectrum of the membranes (spectrum not shown), is due to some small amount of denatured cytochrome *b*.

Resolution of Cytochrome Component Spectra. Preliminary resolved spectra of the cytochrome components had been obtained in a previous study (Koland et al., 1984) in which an RMI method was used to extract the reduced-minus-oxidized spectra of each cytochrome component from a set of spectra of the cytochrome *d* complex at different solution potentials. However, this earlier computation was done with an oxidase sample with an abnormally low absorbance ratio of 1.3 for the cytochrome *b* peak at 560 nm (A_{560}–A_{577}) to the cytochrome *d* peak at 628 nm (A_{628}–A_{607}) in the reduced-minus-oxidized spectrum. This value is significantly lower than the value (1.8) of the typical preparations of the cytochrome *d* complex used in this study. New sets of resolved spectra were computed from data of potentiometric titrations performed with either 50 mM octyl glucoside [as before, Koland et al. (1984)] or 25 mM sodium cholate. As the latter detergent further separates the midpoint potentials of the three cytochrome components (Lorence et al., 1984), a better resolution of spectra could be obtained. The reduced-minus-oxidized spectra of all three cytochrome components of the cytochrome *d* complex were

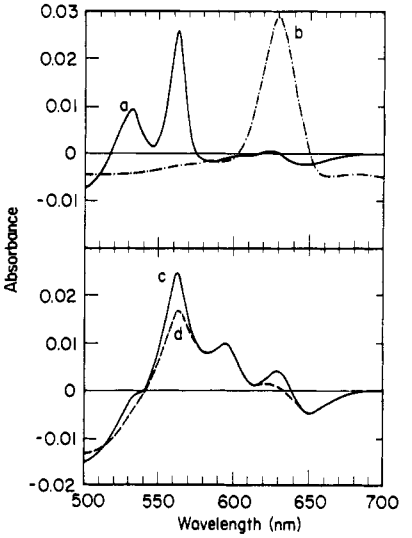

FIGURE 4: Generation of the spectra of the cytochrome components in the cytochrome *d* complex by the spectral subtraction method. The spectrum (a) of the cytochrome b_{558} was obtained by subtracting the spectrum of the sample at −50 mV from the spectrum of the sample at −76 mV. The spectrum (b) of cytochrome *d* was obtained by subtracting the spectrum of the sample at +205 mV from the spectrum taken at +185 mV. The difference in spectra at +78 and +40 mV yielded largely the cytochrome b_{595} spectrum (c). The interference due to cytochrome b_{558} in the cytochrome b_{595} spectrum was eliminated by subtracting enough of the cytochrome b_{558} spectrum to eliminate the β-band at 531 nm. Finally, the cytochrome *d* interference at 628 nm was eliminated (spectrum d) from the cytochrome b_{595} spectrum by using the Nernst equation assuming an $N = 1$ component. This set of data is taken from a potentiometric titration using 25 mM sodium cholate and 0.1 M potassium phosphate, pH 8.0, in the titration solution.

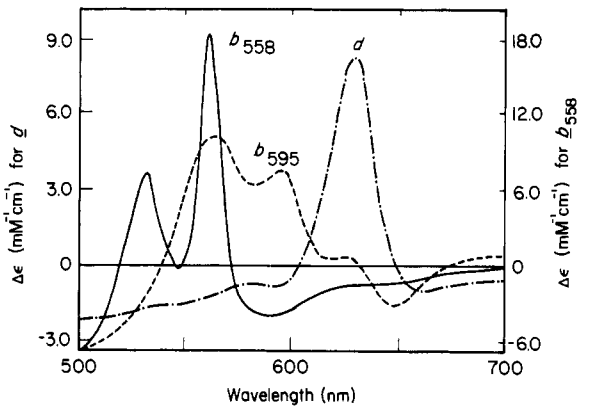

FIGURE 5: Resolved spectra of the cytochrome components from potentiometric titration data of the purified cytochrome *d* complex analyzed with the reiterative matrix inversion method. Shown are the reduced-minus-oxidized spectra attributed to cytochromes b_{558} (—), b_{595} (- - -), and *d* (- · -). The extinction coefficients are based on values from coulometric titrations of cytochrome *d* (Figure 1B). See text for details.

obtained (Figure 4) by subtracting spectra recorded at different potentials.

The simple spectral subtraction method yielded component spectra similar to those obtained by using the RMI method (Figures 4 and 5). In either case the contributions of the resolved spectra to the composite spectrum were determined by a least-squares analysis and generated the same set of results. Figure 5 shows the results obtained by using the RMI method.

Qualitatively, the set of results presented here (Figure 5) is similar to the one obtained previously (Koland et al., 1984). The major difference is that this new set of results (Figure 5) shows a larger relative contribution due to cytochrome b_{558}.

Table I: Quantitative Analysis of the Cytochromes in the Purified Cytochrome *d* Oxidase Complex

method	amount per complex[a]			
	d	b_{558}	total *b*	total iron
(1) coulometry	1.4 ± 0.3		2.3 ± 0.3[b]	3.7 ± 0.5[c]
(2) CO-binding titration	1.6 ± 0.1			
(3) resolved spectra, using extinction coeff of pure b_{558} α-band		0.8 ± 0.2[d]		
(4) extinction coeff of β-band of pure b_{558}		0.9 ± 0.1[e]		
(5) heme extraction/pyridine hemochromogen			1.9 ± 0.3	
(6) neutron activation				3.7 ± 0.2[f]
(7) atomic absorption				2.7 ± 0.2[f]
(8) colorimetric analysis				3.3 ± 0.8[f]

[a] Amount of cytochrome per complex is based on a molecular weight of 100 000 for the complex based on the assumption of a 1:1 complex of the two subunits with the molecular weights deduced from the DNA sequence of the gene (R. B. Gennis, unpublished results). [b] A value of 2.1 ± 0.3 was obtained from the cytochrome *d* complex purified from a ubiquinone-lacking strain AN386. [c] Assuming total iron equals total cytochrome since there is no non-heme iron. [d] $\Delta\epsilon_{560-578nm,red-oxid}$ of 22 mM^{-1} cm^{-1} for the purified cytochrome b_{558} was used to estimate the amount of cytochrome b_{558} in its resolved spectrum (Figure 5). [e] The extinction coefficient of the β-band of the purified cytochrome b_{558} ($\Delta\epsilon_{531nm,red-oxid}$, Figure 7) was used to quantitate the amount of cytochrome b_{558} in the oxidase complex (Figure 7) by using the reduced-minus-oxidized spectrum of the oxidase complex. This method, unlike the previous one, does not employ the use of any resolved spectral data of Figure 5. [f] These values are obtained from the work of Miller & Gennis (1983).

Cytochrome b_{558} showed α- and β-bands at 560 and 531 nm, respectively. The cytochrome *d* spectrum has a major α-band at 628 nm. The cytochrome b_{595} spectrum is complex with a β-band at 562 nm and an attenuated α-band at 595 nm and a trough at 645 nm. The sum of these three spectra associated with cytochromes b_{558}, b_{595}, and *d* is shown in Figure 6, along with the difference spectrum of the terminal oxidase complex. The good agreement in the region of 500–645 nm is an indication of the spectral resolution data in this region.

Assignments of Extinction Coefficients and Estimates of the Amounts of Cytochromes b_{558} and b_{595}. In the CO-binding titration, a cytochrome *d* complex sample with a $\Delta A_{628-607nm,red-oxyg}$ of 0.075 bound 81 nmol of carbon monoxide in the 8-mL anaerobic cuvette (Figure 2). This result yields an extinction coefficient of 7.4 mM^{-1} cm^{-1} for the wavelength pair 628 and 607 nm in the reduced-minus-oxygenated spectrum for cytochrome *d* in the purified complex. The estimated extinction coefficient obtained by coulometry was similar, 8.0 mM^{-1} cm^{-1} for this wavelength pair.

Figure 5 shows estimated extinction coefficients for the resolved spectra of the three components in the complex. The ordinate on the left is calculated from the coulometric analysis of the cytochrome *d* component. The relative absorption intensities are obtained by the spectral resolution of the reduced-minus-oxidized spectrum of the purified complex. The extinction coefficients for the b_{558} and b_{595} components can be estimated by assuming a 1:1 stoichiometry with heme *d*, with the left-hand ordinate, or by assuming a 1:2 stoichiometry relative to heme *d*, with the right-hand ordinate. The extinction coefficient of purified cytochrome b_{558} has been independently measured as 22 mM^{-1} cm^{-1} for the wavelength pair 560–580 nm (Green et al., 1986). This is in reasonable agreement with the assumption of one b_{558} per complex (1:2 ratio of b_{558}:*d*), using the ordinate on the right-hand side of Figure 5.

Additional support for this estimate comes from data independent of the resolved spectral data. One can simply use the β-band at 531 nm of cytochrome b_{558} to quantify the amount of this cytochrome in the cytochrome *d* complex, because only the cytochrome b_{558} component has a peak at this wavelength (Figure 5). Since the cytochrome b_{595} component spectrum takes the shape of a quadratic curve in the region of 500–550 nm (within a 5% error), elimination of the spectral interference due to this cytochrome can be performed by fitting a base-line curve to a quadratic equation as described under Materials and Methods. When the pyridine hemochromogen analysis was used, the β-band at 531 nm of the purified cytochrome b_{558} was found to have a $\Delta\epsilon$ = 9.2 mM^{-1} cm^{-1} (Figure 7A). Using the dithionite reduced minus ferricyanide oxidized spectrum of the purified cytochrome *d* complex and the extinction coefficient $\Delta\epsilon_{628-610,red-oxyg}$ for cytochrome *d* of 7.4 mM^{-1} cm^{-1} found by CO-binding titration, one can estimate that there is 0.53 molecule of cytochrome b_{558} per molecule of cytochrome *d*. The CO-binding result rather than that from coulometry was used as the better estimate of the extinction coefficient for cytochrome *d* because the CO-binding method had a smaller standard deviation and was therefore judged to be more precise. This result again indicates that there is one molecule of cytochrome b_{558} per complex (Table I). With two molecules of total protoheme IX per complex, this would indicate that there is one molecule of cytochrome b_{595} per complex.

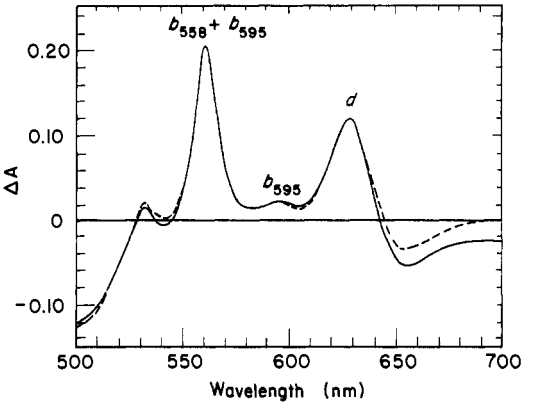

FIGURE 6: Summation of component spectra. The component spectra of the resolved cytochrome components (Figure 5) were numerically summed to reconstruct the composite reduced-minus-oxidized spectrum of the cytochrome *d* complex (---). The authentic electrodically generated reduced-minus-oxidized spectrum of the cytochrome *d* complex (—) is shown for comparison. This latter spectrum is the difference in spectra of the cytochrome *d* complex measured at −100 and +375 mV.

A summary of extinction coefficients for assays of cytochrome b_{558} and *d* is given in Table II. Use of the extinction coefficient of the β-band at 531 nm of cytochrome b_{558} should allow the amount of this cytochrome to be estimated in the presence or absence of cytochrome b_{595}.

Spectral Comparison of Cytochrome b_{595} with Cytochrome c Peroxidase. The spectra of cytochrome b_{595} and of cytochrome *c* peroxidase are compared in Figure 8. Heme extraction and pyridine hemochromogen analysis was used to assign extinction coefficients to the spectrum of cytochrome

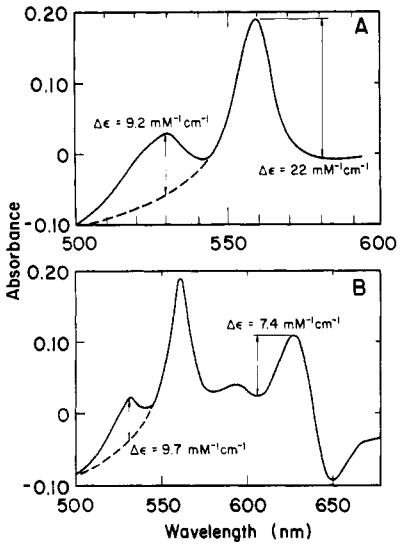

FIGURE 7: Use of the β-band at 531 nm of the cytochrome b_{558} to quantify the amount of cytochrome b_{558} in the cytochrome d complex. In (A), the dithionite reduced minus ferricyanide oxidized spectrum of the purified cytochrome b_{558} (Green et al., 1985) is shown. In (B) the dithionite reduced-minus-oxygenated spectrum of the purified cytochrome d complex is shown. Because the peak at 531 nm is solely due to the cytochrome b_{558} component in the cytochrome d complex (Figure 5), one can use this peak to quantify cytochrome b_{558} in the complex. Spectral interference due to cytochrome b_{595} in this region can be eliminated by fitting a base-line curve (---) to a quadratic equation as described under Materials and Methods. In (A), the extinction coefficient for the difference in the peak at 531 nm and this quadratic base line is found to be 9.2 mM^{-1} cm^{-1} for the purified cytochrome b_{558} by using pyridine hemochromogen analysis. In (B), the extinction coefficient of the cytochrome d peak (628 vs. 607 nm) has been found to be 7.4 mM^{-1} cm^{-1} by CO-binding titrations (Figure 3). Using this cytochrome d extinction coefficient along with the extinction coefficient of 9.2 mM^{-1} cm^{-1} for the β-band of cytochrome b_{558} found in (A), one can estimate that there is 0.53 molecule of cytochrome b_{558} per molecule of cytochrome d.

Table II: Recommended Extinction Coefficients for Assays of Cytochrome b_{558} and Cytochrome d

	cytochrome	extinction coefficient
1	cytochrome b_{558}	$\Delta\epsilon_{560-580nm,red-oxid}$ = 22.0 mM^{-1} cm^{-1} [a]
	cytochrome b_{558}	$\Delta\epsilon_{531nm,red-oxid}$ = 9.2 mM^{-1} cm^{-1} [b]
2	cytochrome d	$\Delta\epsilon_{628-607nm,red-oxyg}$ = 7.4 mM^{-1} cm^{-1} [c]

[a] Cytochrome b_{595} interferes with this determination. [b] This value is for the height of the β-band at 531 nm of cytochrome b_{558} as described under Materials and Methods. Quantifying cytochrome b_{558} was done by using a purified sample of this cytochrome (Green et al., 1985) and pyridine hemochromogen analysis. This value is independent of the amount of cytochrome b_{595}. [c] This value was found from CO-binding titration data and is for the reduced-minus-oxygenated spectrum.

c peroxidase. The value obtained at 560 nm was 5.3 mM^{-1} cm^{-1}. There is no independent estimate of the extinction coefficient for cytochrome b_{595}, and the spectrum in the figure has been adjusted in magnitude to facilitate comparison. Both reduced-minus-oxidized spectra show a predominant β-band at 560 nm, an attenuated α-band between 590 and 600 nm, and a trough near 650 nm. The similarity suggests that cytochrome b_{595} has the same prosthetic group as does cytochrome c peroxidase, a high-spin protoheme IX. If there is one b_{595} per complex, the extinction coefficient at 560 nm would be 9.8 mM^{-1} cm^{-1}, nearly double that of cytochrome c peroxidase (see Figure 5).

Discussion

The identity of the species giving rise to the 595-nm peak in the reduced-minus-oxidized spectrum of the *E. coli* membranes and of the purified cytochrome d complex has been a

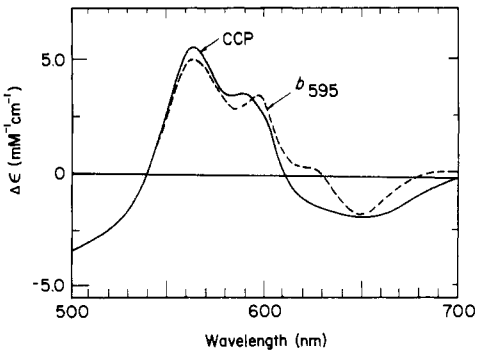

FIGURE 8: Comparison of the dithionite reduced minus ferricyanide oxidized spectra of cytochrome c peroxidase (—) and the resolved cytochrome b_{595} component (---). An extinction coefficient of 5.3 mM^{-1} cm^{-1} at 560 nm for cytochrome c peroxidase was found by recording the reduced-minus-oxidized spectrum and then extracting heme and performing a pyridine hemochromogen to determine the amount of protoheme IX. The resolved spectrum of cytochrome b_{595} was taken from Figure 5, and the amplitude has been adjusted to facilitate comparison. See text for details.

problem for some time. This 595-nm peak has long been assigned as cytochrome a_1 by virtue of the position of the absorption band. This assignment, however, has been made with no convincing evidence for the presence of heme a in *E. coli* except for the report (Lemberg et al., 1955) that porphyrin a can be extracted in trace amounts. The significance of this 595-nm peak in pure preparations of the cytochrome d complex has been both ignored in *Photobacterium phosphorium* (Watanabe et al., 1979) and attributed to an impurity in the case of the *E. coli* cytochrome d complex (Kita et al., 1984b). However, in the initial report (Miller & Gennis, 1983) of the purification of the cytochrome d complex from *E. coli*, it was found that the relative amounts of cytochromes a_1 and d were approximately the same both in the pure material and in membranes. This indicated that cytochrome a_1 was not an impurity in the preparation. Also, at that time, it was observed that no heme a could be extracted from the purified cytochrome d complex (Miller & Gennis, 1983; Koland et al., 1984). The possibility that the cytochrome a_1 component might contain protoheme IX rather than heme a as its prosthetic group was suggested by the potentiometrically resolved cytochrome a_1 component spectrum (Koland et al., 1984). The spectrum obtained resembled the spectra of proteins with high-spin protoheme IX such as tryptophan 2,3-dioxygenase (Ishimura et al., 1967; Koike & Feigelson, 1971) and cytochrome c peroxidase (Yonetani & Ray, 1965) which in their reduced state showed an absorbance peak at 590 nm.

The results of this report serve as additional evidence that the prosthetic group of cytochrome a_1 is most likely high-spin protoheme IX and that it should be renamed as cytochrome b_{595}. This is indicated by the observations that (1) extracted protoheme IX is sufficient to fully account for the sum of the cytochrome b_{558} and b_{595} (Table I) and (2) the resolved reduced-minus-oxidized spectrum of cytochrome b_{595} is very similar to that of the protoheme IX containing cytochrome c peroxidase (Figure 7).

The recent reports (Baines et al., 1984; Poole et al., 1984) concerning a partially purified soluble cytochrome a_1 of *E. coli* include the observation that it contains protoheme IX rather than heme a. Consequently, Poole et al. (1984) renamed this cytochrome a_1 as haemoprotein b_{590}. This soluble protoheme IX containing protein has a spectrum similar to that of the cytochrome b_{595} component of the cytochrome d complex that is presented here.

The proposal that cytochrome b_{595} contains protoheme IX has implications concerning the nature of prosthetic groups of other cytochromes a_1. Of the cytochromes a_1 listed in a recent survey (Poole, 1983), only a few have actually been shown to contain heme *a*. Many of them may indeed contain high-spin protoheme IX as their prosthetic group. Kranz & Gennis (1985) have already shown immunological cross-reactivity of the cytochrome *d* complex of *E. coli* to such Gram-negative bacteria as *Enterobacter aerogenes*, *Photobacterium phosphorium*, *Acinetobacter H01N*, and *Azotobacter vinelandii*. If further analogy holds, the cytochrome a_1 components of these species may also contain protoheme IX rather than heme *a*.

The data in this paper (Table I) indicate that the cytochrome *d* complex molecule contains two cytochrome *d* centers (heme *d*), one cytochrome b_{595} center (high-spin protoheme IX), and one cytochrome b_{558} center (low-spin protoheme IX). The major inconsistency with this conclusion is the amount of iron measured in the complex. The value of 2.7 iron atoms per complex obtained by atomic absorption spectroscopy may have been a low estimate. Other methods for estimating iron gave values of 3.3 and 3.7 iron atoms per complex (Miller & Gennis, 1983). With estimates that there are two molecules of cytochrome *d* per complex (from coulometry and CO-binding titrations) and that there are two molecules of *b*-type cytochrome per complex (from heme extraction and coulometry), the value of four iron atoms per complex is expected.

Interpretation of the data in Table I indicates with good assurance that there is 1 mol of cytochrome b_{558} per 2 mol of cytochrome *d*. The calculated stoichiometry per mole of enzyme complex depends on the molecular weight of the complex. The best evidence (unpublished results) is based on the DNA sequencing of the genes coding for the two subunits, indicating molecular weights of 57 000 and 43 000. In addition, quantitative N-terminal analyses show a 1:1 ratio of the two subunits in the purified complex (Hermodson and Gennis, unpublished results). Earlier experiments (Kranz & Gennis, 1984) suggesting that the lower molecular weight subunit is present as a dimer have not been reproducible with fresh preparations of the purified complex. Hence, the best interpretation at present is that the enzyme is an $\alpha\beta$ heterodimer with a molecular weight of 100 000. The data in Table I, therefore, show 0.85 mol of cytochrome b_{558} and 1.6 mol of cytochrome *d* per mole of complex. Possibly these numbers are somewhat low due to loss of heme from a portion of the enzyme during the preparation. The stoichiometry of cytochrome b_{595} can be calculated by subtraction, assuming that all the protoheme IX is accounted for by the sum of the b_{558} and b_{595} components. This indicates about 1.1 mol of b_{595} per mole of enzyme. This predicts a maximum of four iron atoms per enzyme, which is in reasonable agreement with the iron analyses, except for the value obtained by atomic absorption, which apparently gives a low estimate.

It is noted that Kita et al. (1984b) estimated one cytochrome b_{558} and one cytochrome *d* per complex. However, their preparation (12.3 nmol of protoheme IX/mg) of the complex appears to have lost more heme than that used in the present work [18.9 nmol of protoheme IX/mg (Miller & Gennis, 1983)]. It is important to realize, however, that there is some ambiguity in the data, especially with regard to stoichiometry of the cytochrome b_{595} component, which will require additional work to confirm.

The role played by the cytochrome b_{595} component is not clear. It does not appear to bind CO at stoichiometric levels of CO on the basis of the data presented in this work and by the lack of dependence of the electrochemical midpoint on CO (Lorence et al., 1984a). This conflicts with earlier data suggesting that this cytochrome binds CO (Edwards et al., 1981; Poole et al., 1981). Thus, the CO-binding results presented here indicate that cytochrome b_{595} may not be involved in ligand binding, but rather it may have an electron-transfer function. On the other hand, the spectroscopic similarity between cytochrome b_{595} and the peroxidase/catalase type of centers certainly suggests a role in ligation of oxygen. Further studies will be directed at this issue of cytochrome b_{595} function.

Registry No. Cytochrome *d*, 9035-36-3; cytochrome b_{558}, 9064-78-2; cytochrome a_1, 9035-35-2; cytochrome b_{595}, 98420-31-6; oxidase, 9035-73-8.

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D-Lactate Oxidation and Generation of the Proton Electrochemical Gradient in Membrane Vesicles from *Escherichia coli* GR19N and in Proteoliposomes Reconstituted with Purified D-Lactate Dehydrogenase and Cytochrome *o* Oxidase

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ABSTRACT: The respiratory chain in the cytochrome *d* deficient mutant *Escherichia coli* GR19N is a relatively simple, linear system consisting of primary dehydrogenases, ubiquinone 8, cytochrome *b*-556, and cytochrome *o* oxidase. By use of right-side-out and inside-out membrane vesicles from this strain, various oxidase activities and the generation of the H^+ electrochemical gradient were studied. Oxidation of ubiquinol 1 or *N*,*N*,*N'*,*N'*-tetramethyl-*p*-phenylenediamine, which donate electrons directly to the terminal oxidase, generates a H^+ electrochemical gradient comparable to that observed during D-lactate oxidation. In contrast, D-lactate/ubiquinone 1 or D-lactate/ferricyanide oxidoreductase activity does not appear to generate a membrane potential, suggesting that electron flow from D-lactate dehydrogenase to ubiquinone is not electrogenic. Moreover, proteoliposomes reconstituted with purified D-lactate dehydrogenase, ubiquinone 8, and purified cytochrome *o* catalyze D-lactate and ubiquinol 1 oxidation and generate a H^+ electrochemical gradient similar to that observed in membrane vesicles. Strikingly, in inside-out vesicles, NADH oxidation generates a H^+ electrochemical gradient that is very significantly greater than that produced by either D-lactate or ubiquinol 1; furthermore, NADH/ubiquinone 1 and NADH/ferricyanide oxidoreductase activities are electrogenic. It is suggested that the only component between D-lactate dehydrogenase or ubiquinol and oxygen in GR19N membranes that is directly involved in the generation of the H^+ electrochemical gradient is cytochrome *o*, which functions as a "half-loop" (i.e., the oxidase catalyzes the scalar release of 2 H^+ from ubiquinol on the outer surface of the membrane, vectorial transfer of 2 e^- from the outer to the inner surface, and scalar utilization of 2 H^+ on the inner surface to reduce oxygen). In contrast, between NADH and oxygen, generation of the H^+ electrochemical gradient occurs at two sites, one between NADH dehydrogenase and ubiquinone by an unspecified mechanism and the other at cytochrome *o* oxidase.

A compelling body of evidence has now accumulated demonstrating that many energy-coupling membrane systems generate a large H^+ electrochemical gradient ($\Delta\bar{\mu}_{H^+}$)[1] that is the immediate driving force for a wide array of phenomena [cf. Skulachev and Hinkle (1981), Nichols (1982), and Kaback (1986) for recent reviews]. However, the mechanism by which $\Delta\bar{\mu}_{H^+}$ is generated, particularly during respiration, is still conjectural and often controversial.

In aerobically growing *Escherichia coli*, like mitochondria, $\Delta\bar{\mu}_{H^+}$ is generated primarily by substrate oxidation via a membrane-bound respiratory chain with oxygen as terminal electron acceptor. Even in this relatively simple respiratory system, neither the site(s) of $\Delta\bar{\mu}_{H^+}$ generation nor the mechanism has been resolved [cf. Ingledew and Poole (1984) for a review]. In general, three types of mechanisms have been proposed to explain respiration-driven $\Delta\bar{\mu}_{H^+}$ generation: (i) "Loops". According to this notion, the electron and H^+ carriers that comprise the respiratory chain are disposed alternatively and asymmetrically across the membrane in such a manner that H^+ moves from one side of the membrane to the other with a net flux of electrons in the opposite direction (Mitchell, 1966, 1968). (ii) "Pumps". By this means, a specific intermediate in the respiratory chain (e.g., the mitochondrial terminal oxidase) acts as a primary H^+ pump during oxida-

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[1] Abbreviations: $\Delta\bar{\mu}_{H^+}$, the proton electrochemical gradient; $\Delta\Psi$, membrane potential; ΔpH, pH gradient; octyl glucoside, octyl β-D-glucopyranoside; PMS, phenazine methosulfate; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; TPP^+, tetraphenylphosphonium; diS-C_3-(5), 3,3'-diisopropylthiodicarbocyanine; diBA-C_2-(5), bis(1,3-diethylbarbituric acid) pentamethine oxonol; TMPD, *N*,*N*,*N'*,*N'*-tetramethyl-*p*-phenylenediamine; DCI, dichlorophenolindophenol; HQNO, 2-heptyl-4-hydroxyquinoline *N*-oxide; *p*CMBS, *p*-(chloromercuri)benzenesulfonate; BAL, 2,3-dimercapto-1-propanol; RSO, right side out; ISO, inside out; KP_i, potassium phosphate; Q_1H_2, ubiquinol 1; DTT, dithiothreitol; D-L-DH, D-lactate dehydrogenase; NADH, reduced nicotinamide adenine dinucleotide.

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