Doolittle, R. F., Woodbury, N. W., & Jue, R. A. (1982) *Biosci. Rep. 2*, 405–412.
Giorginis, S., & Subramanian, A. R. (1980) *J. Mol. Biol. 141*, 393–408.
Guerrier-Takada, C., Subramanian, A. R., & Cole, P. E. (1983) *J. Biol. Chem. 258*, 13649–13652.
Kumura, M., Foulaki, K., Subramanian, A. R., & Wittmann-Liebold, B. (1982) *Eur. J. Biochem. 123*, 37–53.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Laughrea, M., & Moore, P. B. (1978) *J. Mol. Biol. 121*, 411–430.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
McKay, D. B., & Steitz, T. A. (1981) *Nature (London) 290*, 744–749.
McPherson, H., Jurnak, F., Wang, A., Kolpak, F., Rich, A., Molineux, I., & Fitzgerald, P. (1980) in *Biophysical Discussions: Proteins and Nucleoproteins*, pp 155–173, Rockefeller University Press, New York.
Minks, M. A., Suryanarayana, T., & Subramanian, A. R. (1978) *Eur. J. Biochem. 82*, 271–277.
Schnier, J., Kumura, M., Foulaki, K., Subramanian, A. R., Isono, K., & Wittmann-Liebold, B. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 1008–1011.
Smolarsky, M., & Tal, M. (1970) *Biochim. Biophys. Acta 199*, 447–452.
Stark, G. R. (1977) *Methods Enzymol. 67*, 129–132.
Stöffler, G., & Wittmann, H. G. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 2283–2287.
Subramanian, A. R. (1975) *J. Mol. Biol. 95*, 1–8.
Subramanian, A. R. (1980) *J. Biol. Chem. 255*, 3227–3229.
Subramanian, A. R. (1983) *Prog. Nucleic Acid Res. Mol. Biol. 28*, 101–142.
Subramanian, A. R., & Mizushima, S. (1979) *J. Biol. Chem. 254*, 4309–4312.
Subramanian, A. R., Haase, C., & Giesen, M. (1976) *Eur. J. Biochem. 67*, 591–601.
Subramanian, A. R., Rienhardt, P., Kimura, M., & Suryanarayana, T. (1981) *Eur. J. Biochem. 119*, 245–249.
Subramanian, A. R., Steinmetz, A., & Bogorad, L. (1983) *Nucleic Acids Res. 11*, 5277–5286.
Suryanarayana, T., & Subramanian, A. R. (1979) *J. Mol. Biol. 127*, 41–54.
Suryanarayana, T., & Subramanian, A. R. (1983) *Biochemistry 22*, 2715–2719.
Szer, W., & Leffler, S. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 3611–3615.
Thomas, J. O., Boublik, M., Szer, W., & Subramanian, A. R. (1979) *Eur. J. Biochem. 102*, 309–314.
Wittmann, H. G. (1982) *Annu. Rev. Biochem. 51*, 155–183.
Wittmann-Liebold, B., Ashman, K., & Dzionara, M. (1983) *FEBS Lett. 154*, 31–41.
Zamir, A., Miskin, R., Vogel, Z., & Elson, D. (1974) *Methods Enzymol. 30*, 406–426.

Potentiometric Analysis of the Purified Cytochrome *d* Terminal Oxidase Complex from *Escherichia coli*†

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ABSTRACT: The cytochrome *d* terminal oxidase complex is a principal component of the aerobic respiratory chain of *Escherichia coli*. This purified complex contains two polypeptides as analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and the reduced minus oxidized spectrum indicates that cytochromes *b*-558, a_1, and *d* are all components of the complex. Changes in the absorption spectrum of the complex between 500 and 700 nm were analyzed as a function of the solution electrochemical potential. A spectral resolution algorithm was used to extract the reduced minus oxidized spectrum of each electrochemically active species from the set of spectra of the complex at different solution potentials. The procedure yielded reduced minus oxidized difference spectra for cytochromes *b*-558, a_1, and *d*, along with the midpoint potentials and Nernst *n* values for each cytochrome. The midpoint potentials (E_m) and *n* values for the solubilized complex were as follows: cytochrome *b*-558, E_m = 61 mV and n = 0.8; cytochrome a_1, E_m = 113 mV and n = 1; cytochrome *d*, E_m = 232 mV and n = 1. The spectrum of cytochrome *b*-558 was typical of *b*-type cytochromes, and that of cytochrome *d* was dominated by a band centered at 628 nm. The difference spectrum of cytochrome a_1 indicated an α band at 594 nm, a strong β band near 560 nm, and a trough near 645 nm. The spectrum is similar to the spectra of high-spin heme *a* model compounds.

The cytochrome *d* terminal oxidase complex is a principal component of the aerobic respiratory chain of *Escherichia coli* (Miller & Gennis, 1983; Bragg, 1979; Haddock & Jones, 1977). The respiratory chain is branched and contains two terminal oxidases, cytochrome *d* and cytochrome *o* [see Bragg (1979) and Haddock & Jones (1977)]. The cytochrome *d* terminal oxidase is induced when the cells are grown under conditions of oxygen limitation (Kranz & Gennis, 1983), and previous work has shown that the affinity for molecular oxygen of cytochrome *d* is higher than that of cytochrome *o* (Rice & Hempfling, 1978). Both terminal oxidases have been purified to homogeneity (Matsushita et al., 1983; Miller & Gennis, 1983; Kita et al., 1982). The reduced minus oxidized difference spectrum of the cytochrome *d* complex is complicated

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(Miller & Gennis, 1983). Cytochromes *b*-558, a_1, and *d*, which have been identified in the *E. coli* membrane [e.g., see Lorence et al. (1984)], are all present in the complex. Attempts to separate these three cytochromes biochemically have not been successful (Miller & Gennis, 1983; unpublished results), and immunoprecipitation experiments have verified that these three cytochromes are associated in a complex (Kranz & Gennis, 1983).

The cytochrome *d* complex contains two subunits as determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)[1] analysis. Preliminary studies indicated the functional unit may contain one copy of each subunit, though this is still not certain. No prosthetic groups are present other than the iron-containing hemes. Analyses indicate between three and four iron molecules per functional unit, assuming a dimeric structure. The enzyme has been reconstituted with phospholipids to form proteoliposomes, in which the oxidase functions as a ubiquinol 8 oxidase and generates a transmembrane potential across the artificial bilayers (Koland et al., 1984).

The prosthetic group associated with cytochrome *d* is a chlorin. This has been purified and a chemical structure tentatively assigned (Barrett, 1956). It is clear that cytochrome *d* binds to CO and O_2 (Poole et al., 1982, 1983; Castor & Chance, 1959; Pudek & Bragg, 1976b). Until recently, cytochrome a_1 was viewed as a minor cytochrome component in the *E. coli* membrane which might possibly bind to CO (Poole et al., 1981) and possibly function as a third terminal oxidase (Edwards et al., 1981). However, it is now clear that this component is associated with cytochromes *b*-558 and *d* in a complex. The prosthetic group associated with cytochrome a_1 is less certain. The absorption maximum in the reduced minus oxidized spectrum at 595 nm is much smaller in magnitude than the α bands of either cytochrome *b*-558 or cytochrome *d*. It has been reported that porphyrin *a* can be extracted from *E. coli* (Lemberg et al., 1955), but the amounts were small, and the porphyrin was not purified.

The application of potentiometric methods to biochemical systems has been reviewed (Hendler et al., 1975; Wilson, 1978; Dutton, 1978), and potentiometric analyses of the cytochrome population of the *E. coli* electron-transport system have been documented (Pudek & Bragg, 1976a; Reid & Ingledew, 1979; Hendler & Shrager, 1979; Van Wielink et al., 1982).

In this paper, the absorption spectrum of the cytochrome *d* complex was monitored as a function of the solution electrochemical potential. The data were used to determine the reduced minus oxidized spectrum of each electrochemically active species, i.e., cytochromes *b*-558, a_1, and *d*. The spectrum obtained for the cytochrome a_1 component is similar in shape to that deduced for cytochrome a_3 of the mitochondrial cytochrome *c* oxidase (Vannest, 1966) and to the spectra of high-spin heme *a* model compounds (Carter & Palmer, 1982). However, efforts to chemically extract heme *a* or porphyrin *a* from the complex have thus far been unsuccessful.

Experimental Procedures

Materials

The following materials were supplied by the indicated firms: cholic acid, benzylviologen, phenazine ethosulfate, phenazine methosulfate, 5-hydroxy-1,4-naphthoquinone, and *N,N*-dimethyl-*p*-phenylenediamine (Sigma Chemical Co.); ferrocene and 2-hydroxy-1,4-naphthoquinone (Aldrich Chemical Co.); 1,2-naphthoquinone (ICN Pharmaceuticals); octyl β-D-glucopyranoside (Calbiochem-Behring); quinhydrone (Eastman Kodak Co.); sodium ferricyanide (Pfaltz and Bauer, Inc.).

Cholic acid was recrystallized from 70% ethanol before use.

Methods

Enzyme Preparation. The preparation of the cytochrome *d* terminal oxidase complex has been recently described (Miller & Gennis, 1983). The specific heme *b* content of the terminal oxidase used in these studies was approximately 18 nmol/mg of protein, using the extinction coefficient for cytochrome *b*-558 determined by Miller & Gennis (1983).

Protein Determinations. A modified procedure of Lowry et al. (1951) was used to determine protein concentrations as previously described (Miller & Gennis, 1983).

Spectrophotometrically Monitored Potentiometric Titrations. (A) Electrochemical Cell. An electrochemical cell incorporating a platinum gauze working (indicating) electrode and Ag/AgCl reference and auxiliary electrodes was constructed. All elements contacting the cell electrolyte were inserted through a Teflon cap which fitted snugly into a 1-cm path-length seamless Pyrex cuvette (J and S Scientific, Crystal Lake, IL). The apparatus was constructed for performing both potentiometric and coulometric studies.

To construct the working electrode, a piece of platinum gauze (20 × 40 mm, 52 mesh) was folded lengthwise into a double layer and fashioned into a square cylinder. Two platinum wire (26-gauge) masts were spot-welded to the electrode. The masts were inserted through holes in the Teflon cap, and gold pin connectors were silver-soldered to the protruding ends.

Reference and auxiliary electrodes were helices of silver wire (8 cm, 24 gauge) silver-soldered to gold pin connectors and electroplated with AgCl (Sawyer & Roberts, 1974). The AgCl electrodes contacted a 4 M potassium chloride electrode solution saturated with AgCl. The auxiliary electrode was inserted through a rubber septum and held either in a 1-cm fine-fritted Büchner funnel with a 4-mm Pyrex stem or in a 3-mL plastic syringe barrel coupled via vinyl tubing to a 4-mm glass stem. The stem of the auxiliary electrode was filled with 4% agar in 0.1 M potassium phosphate–0.2 M potassium chloride, pH 7.0, and inserted through the Teflon cap to contact the cell electrolyte.

Two different reference electrode configurations were employed. In one case, the reference electrode was held in a glass vessel external to the cell, and a three-way stopcock was used as a liquid junction. The electrode was connected to the cell by a vinyl tube terminating in a polyethylene capillary. The capillary was inserted through the Teflon cap to contact the cell electrolyte. Both the connection tube and the capillary were filled with the electrolyte solution. To reduce electrical noise, the connection tube was shielded with copper braid.

In the second case, the Ag/AgCl reference electrode was held in a 3-mL plastic syringe barrel mounted on top of the cell. This electrode was connected via a polyethylene capillary to the cell electrolyte. This capillary was filled with agar to prevent excessive flow of liquid through the junction. The second reference electrode configuration proved to evoke much less electrical noise. The Ag/AgCl electrodes were calibrated with a standard calomel electrode (Radiometer-Copenhagen) and were within 2 mV of the literature potential (199 mV).

The cell was stirred by a 3-mm glass rod in the form of an auger. The rod extended through the Teflon cap and was coupled via a short silicone rubber tube to a 3-V electric motor. The cell was purged constantly with argon gas to prevent the entry of oxygen. Ultrapure argon gas (Air Products Corp.)

[1] Abbreviation: SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

was passed through heated copper turnings (475 °C) to remove residual oxygen and was transported via copper tubing to the cell.

(*B*) *Instrumentation.* A Varian-Cary 219 spectrophotometer with a thermostated cell compartment, held at 25 °C during the experiment, was used to record spectra during potentiometric titrations. A current-measuring, three-electrode potentiostat was constructed (Koland, 1983). Both the potentiostat and spectrophotometer were interfaced with an LSI-11 minicomputer equipped with a real time clock and a dual digital to analog/analog to digital converter (Data Translations), a parallel interface module (constructed by Electronic Services Department, School of Chemical Sciences, University of Illinois, Urbana), and an ADM Model 3A cathode ray tube terminal, retrographics enhanced (Lier Siegler, Inc.). The minicomputer controlled the scanning of the spectrophotometer and sampled absorbance data at 0.5-nm intervals. Spectra were stored on magnetic disks and subsequently plotted on a digital plotter (Houston Instruments).

The desired cell potential (voltage between working and reference electrodes) was software selected and a corresponding voltage signal given of the potentiostat via the digital to analog converter. The cell potential could thus be varied at will by the user at the minicomputer terminal.

(*C*) *Potentiometric Titrations.* Before titrations, the platinum electrode was cleaned by soaking sequentially in 50% aqua regia, concentrated ammonium hydroxide, and distilled water. The electrochemical cell was then filled with the cell electrolyte consisting of 0.1 M potassium phosphate, 0.2 M potassium chloride, and 50 mM octyl glucoside, pH 7.0. The cell was then crudely deoxygenated by the passage of argon gas over the solution with stirring. The following mediators were then added from ethanol stock solutions (the final concentrations and approximate midpoint potentials, respectively, are indicated): benzylviologen (50 μM, −350 mV); 2-hydroxy-1,4-naphthoquinone (5 μM, −139 mV); 5-hydroxy-1,4-naphthoquinone (5 μM, −5 mV); phenazine ethosulfate (5 μM, 55 mV); phenazine methosulfate (5 μM, 80 mV); 1,2-naphthoquinone (5 μM, 143 mV); benzoquinone (5 μM, 270 mV); dimethyl-*p*-phenylenediamine (5 μM, 371 mV); ferrocene (50 μM, 420 mV). Finally, an aliquot of the terminal oxidase preparation (25–40 nmol of heme *b*) solubilized in 10 mM tris(hydroxymethyl)aminomethane–16 mM sodium cholate, pH 7.3, was added so that the total volume was 2.80 mL. The cell was exhaustively deoxygenated by poising at −300 mV for 1 h and the titration begun.

Under computer control, the cell potential was increased to −100 mV and then to 400 mV in small increments of approximately 10 mV. To rapidly alter the cell potential, a fixed quantity of charge was delivered at the working electrode at a potential ~50 mV higher than that desired. When the system attained equilibrium at each new potential, a spectrum was scanned and stored along with the cell potential on magnetic disks. When the cell potential reached 400 mV, a reductive titration was performed with the cell potential being incrementally decreased from 400 to −100 mV. All cell potentials are referenced to the standard hydrogen electrode and were measured at pH 7.0 and 25 °C.

(*D*) *Determination of Midpoint Potentials and Resolution of Spectroscopic Components.* Shrager & Hendler (1982) have discussed in detail the analysis of data from spectrophotometrically monitored potentiometric titrations. The singular value decomposition protocol described by these authors yielded ambiguous results when applied to the cytochrome *d* terminal oxidase titration data. Due to the relatively simple spectral properties of the heme components, a less sophisticated iterative procedure could be applied.

The set of spectra collected during a redox titration were grouped in the form of a matrix, $\mathbf{A}_{nw,np}$, where *n*w is the number of wavelength data points and *n*p is the number of potentials at which spectra were recorded. The corresponding set of potentials, E_i (i = 1, *n*p), are assumed known, as are the midpoint potentials, $E_{m,j}$, and Nernst *n* values, n_j, of each species (j = 1, *n*s) where *n*s is the number of redox-active species contributing to the spectral data.

The fractional reduction of each species j at the potential E_i is given by

$$\mathbf{F}_{ij} = \frac{1}{1 + \exp\{[n_jF/(RT)](E_i - E_{m,j})\}} \quad (1)$$

These fractions form the matrix $\mathbf{F}_{np,ns}$. An additional column of 1's is added to this matrix. The last column of the matrix $\mathbf{F}_{np,(ns+1)}$ corresponds to the instrument base line if difference spectra are recorded or the spectrum of the fully oxidized system if absolute spectra are recorded. The reduced minus oxidized difference spectra of the individual species can also be expressed in matrix form, $\mathbf{D}_{nw,(ns+1)}$, including the base line or spectrum of the fully oxidized form. The following matrix equation then holds:

$$\mathbf{A} = \mathbf{DF}^T \quad (2)$$

Here, the superscript T indicates the matrix transpose operation.

The elements of matrix **F** are estimated by selecting wavelengths or wavelength pairs for which the absorption differences as a function of solution potential largely reflect the fractional reduction of the individual components. The initial wavelengths selected were determined by inspection of the reduced minus oxidized spectrum (Figure 2). The fractional reduction of each species was fit by a nonlinear least-squares algorithm to the Nernst equation (eq 1) to yield values for the midpoint potentials ($E_{m,j}$) and Nernst *n* values (n_i) for each species. By using eq 3, we could determine the component difference spectra, **D**:

$$\mathbf{D} = \mathbf{AF}(\mathbf{F}^T\mathbf{F})^{-1} \quad (3)$$

These spectra were used to choose new wavelength pairs for a more accurate determination of **F**, and the procedure was repeated to yield a new set of difference spectra. The process was iterated until self-consistent results were obtained to yield the final set of titration curves, midpoint potentials, Nernst *n* values, and difference spectra.

It should be noted that this simplified spectral resolution protocol was effective because the spectral overlap of the components was limited and the midpoint potentials of the species involved differed substantially. In less fortuitous cases, a more sophisticated approach would be required [e.g., see Shrager & Hendler (1982)].

Results

The absorption spectra of the cytochrome *d* terminal oxidase solubilized in octyl glucoside are shown in Figure 1. The absolute spectrum of the oxidase prior to deoxygenation is shown in Figure 1 along with the spectrum of the reduced form obtained by poising the deoxygenated oxidase at −100 mV in the potentiostat. The sample was then brought to 375 mV, and the spectrum of the anaerobically oxidized enzyme was recorded. It is obvious that the oxygen-free oxidized form is distinct from the "oxygenated" form of the enzyme, which is the form normally observed after isolation and in the presence of oxygen. For the purposes of this work, it is only important

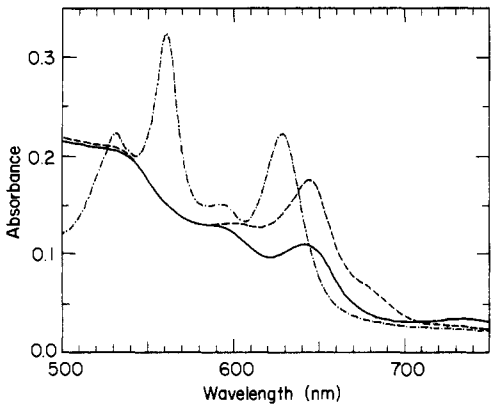

FIGURE 1: Absorption spectra of the cytochrome *d* terminal oxidase complex. The cytochrome *d* terminal oxidase was suspended in 0.1 M potassium phosphate, 0.2 M potassium chloride, and 50 mM octyl β-D-glucoside, pH 7.0, at a concentration of 15 nmol of heme *b*/mL in the electrochemical cell. The mediator titrants, ferrocene (50 μM) and benzylviologen (50 μM), were added to effect oxidation and reduction of the enzyme via the potentiostat (see Experimental Procedures). The spectrum of the purified enzyme was scanned prior to deoxygenation (---). After deoxygenation, the cell was poised at −100 mV, and the spectrum of the reduced enzyme (-·-) was recorded. The cell was then poised at 375 mV, and the spectrum of the anaerobically oxidized system (—) was recorded.

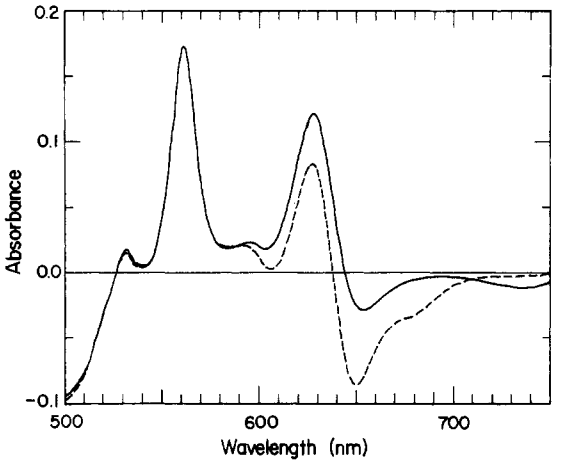

FIGURE 2: Comparison of reduced minus oxidized and reduced minus oxygenated spectra. The reduced, oxidized, and oxygenated spectra of the enzyme given in Figure 1 were used in the construction of the reduced minus oxidized (—) and reduced minus oxygenated spectra (---) shown here.

to note that under anerobic conditions, fully oxidized and reduced forms of the enzyme can be prepared. The potentiometric titrations were performed between these two forms of the enzyme. Figure 2 shows the reduced minus oxidized and reduced minus oxygenated difference spectra of the enzyme. Cytochromes *b*-558, a_1, and *d* are characterized by maxima near 560, 595, and 630 nm, respectively, in these difference spectra. The characterization and interconversions between the anaerobically oxidized and oxygenated forms of this enzyme will be the subject of a future report.

Spectroscopic changes were observed during both reductive and oxidative titrations of the enzyme. The sample was stable under these conditions. The spectroscopic data were analyzed as described under Experimental Procedures. Representative titration profiles for the three resolved cytochrome components are shown in Figure 3. The monitoring wavelengths were selected on the basis of the calculated spectrum of each component (Figure 4) so as to minimize contributions from the other components. For example, the fractional reduction of cytochrome a_1 was monitored at 571 nm because at this

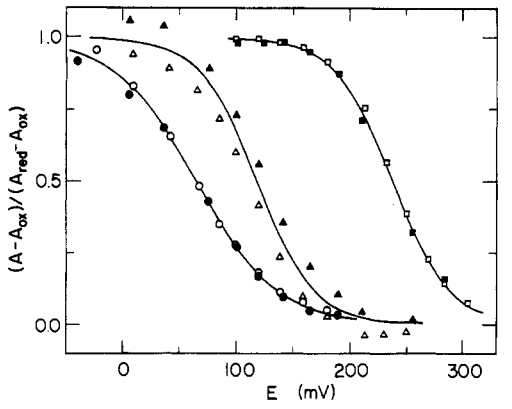

FIGURE 3: Analysis of potentiometric titration data to determine the component midpoint potentials. The spectral data of potentiometric titrations were analyzed as described under Experimental Procedures. Shown are representative titration profiles for the three resolved components: cytochrome *b*-558 (○, ●); cytochrome a_1 (△, ▲); and cytochrome *d* (□, ■). The fractional reduction of these components was determined by measurement of $A_{557}-A_{568}$, A_{571}, and A_{628}, respectively. Open symbols represent the oxidative titration and closed symbols the reductive titration. Also shown are the theoretical curves generated by eq 1 using the midpoint potentials and Nernst *n* values determined by nonlinear least-squares analysis.

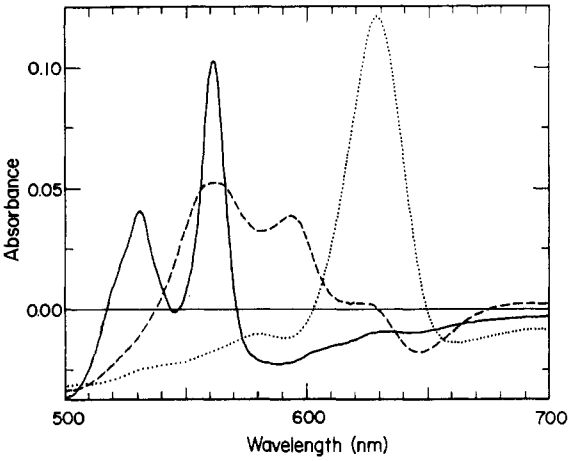

FIGURE 4: Analysis of potentiometric titration data to resolve spectra of the cytochrome components. Analysis of potentiometric titration data was peformed as detailed under Experimental Procedures. Shown are the reduced minus oxidized spectra attributed to cytochromes *b*-558 (—), a_1 (---), and *d* (···).

wavelength the absorbance of cytochrome *b*-558 was constant during the titration (see Figure 4). Both oxidative and reductive titrations were performed, and the data (Figure 3) indicated no serious hysteresis and showed that the system was equilibrated. The slight differences observed for cytochrome a_1 between oxidative and reductive titrations reflect the very small absorption change being monitored in this case and a possible small shift in the spectral base line.

The titration of cytochrome *d* yielded a Nernst *n* value of 1.0 and a midpoint potential of 232 mV. The fit with the theoretical curve to the Nernst equation was excellent (Figure 3). Cytochrome a_1 also titrated as a single component undergoing a one-electron oxidation–reduction reaction with a midpoint potential of 113 mV. The cytochrome *b*-558 component had a midpoint potential of 61 mV and in a series of titrations on different preparations gave *n* values between 0.7 and 0.9. This could reflect either heterogeneity or some negative cooperativity. The variability in the midpoint values among different enzyme preparations was about ±10 mV.

Figure 4 shows the reduced minus oxidized spectra of the three cytochrome components. Note that the molar extinction

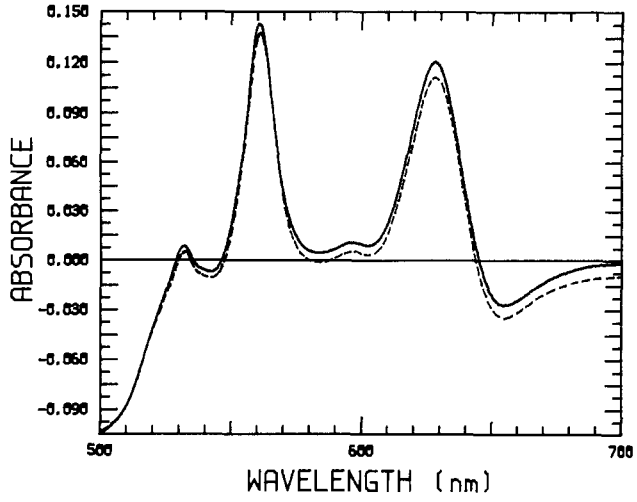

FIGURE 5: Summation of component spectra. The component spectra of Figure 5 were numerically summed to reconstruct the composite reduced minus oxidized spectrum of the terminal oxidase (—). The authentic reduced minus oxidized spectrum of the oxidase (---) is shown for comparison. This spectrum is the difference of the spectra recorded at cell potentials of −100 and 375 mV.

coefficients are not obtained by using this procedure. The heme *b* spectrum shows α and β bands at 561 and 531 nm, respectively. The cytochrome *d* spectrum is quite simple, with a major α band at 628 nm and a very small β band at 580 nm. The spectrum associated with cytochrome a_1 is more complex, with a β band at 562 nm, an attenuated α band at 594 nm, and a trough at 645 nm. The exaggerated α band of the cytochrome a_1 spectrum is not due to incomplete resolution of the cytochrome a_1 and *b* components, as shown by the fact that the sharp β band associated with the *b* cytochrome is totally absent from the spectrum of cytochrome a_1.

The sum of the three spectra associated with cytochromes *b*-558, a_1, and *d* is shown in Figure 5, along with the difference spectrum of the terminal oxidase complex. The good agreement is an indication of the validity of the spectral resolution procedure.

Discussion

In order to understand the mechanism of the cytochrome *d* complex and the nature of the electrogenic reaction that it catalyzes, it is obviously necessary to identify the prosthetic groups and determine their stoichiometry in the functional unit. Potentiometry and spectral resolution techniques were used in this work in order to determine the spectroscopic contributions which each redox-active species makes to the reduced minus oxidized spectrum of the complex.

The first point to be made is that the purified enzyme exists in several forms. Only after oxygen has been removed can the fully reduced and oxidized forms be observed. As isolated, the enzyme is in an oxygenated form which is distinct from the fully oxidized form. Addition of ferricyanide to this oxygenated form has no effect on the spectrum. These properties of the enzyme are not artifacts due to detergent solubilization, and the existence of a stable oxygenated form of cytochrome *d* in *E. coli* membrane preparations has been previously described [e.g., see Pudek & Bragg (1976b) and Poole et al. (1982, 1983)]. It is evident that the potentiometric studies must be performed in samples from which oxygen has been rigorously removed.

The potentiometric studies were performed in the presence of octyl glucoside. Octyl glucoside was selected because in this detergent the midpoint potential of cytochrome *b*-558 is +61 mV, approximately 120 mV lower than it is in the *E. coli* membrane [+180 mV (Lorence et al., 1984)]. This is not the case for all detergents (R. M. Lorence and R. B. Gennis, unpublished results). The lower midpoint potential of this cytochrome in octyl glucoside allows for a much clearer electrochemical resolution of the three cytochromes within the complex, which is an advantage for the procedures utilized in this work. The midpoint potentials of cytochromes a_1 and *d* are only slightly lower in the detergent solution compared to the values determined with membrane preparations (Lorence et al., 1984). A more detailed study is in progress on the effects of detergents as well as pH on the properties of the enzyme.

The data in Figure 3 summarize the reduction of each cytochrome as a function of the solution potential. Both cytochromes a_1 and *d* titrate as simple one-electron components. The data fit well to the Nernst equation with $n = 1.0$. Previous studies have suggested that cytochrome a_1 in the *E. coli* membrane titrates with two midpoint potentials (Reid & Ingledew, 1979). However, this result was probably due to interference of spectroscopic changes in cytochrome *d* (Lorence et al., 1984). Very complicated electrochemical behavior has also been reported for cytochrome *d* (Hendler & Shrager, 1979). The results in this paper, however, very clearly show that cytochrome *d* has relatively simple electrochemical properties, at least in the absence of oxygen.

The titration of cytochrome *b*-558 is also relatively simple, with the only unusual feature being the Nernst *n* value, which is slightly less than 1.0, varying between 0.7 and 0.9. This may be due to heterogeneity resulting from the solubilization and purification protocol. Alternatively, this result could be due to negative cooperativity between *b* cytochromes. Earlier work did indicate that 2 mol of heme *b* is present per 100 000 g of the complex (Miller & Gennis, 1983).

The reduced minus oxidized spectrum of cytochrome *b*-558 which resulted from the spectroscopic resolution appears typical for *b*-type cytochromes (Goldberger et al., 1961; Itagaki & Hager, 1966; Spatz & Strittmatter, 1971). The spectrum derived for cytochrome *d* also appears quite simple. The large trough at 650 nm and the shoulder near 680 nm in the reduced minus oxygenated spectrum of the oxidase complex (Figure 2) are clearly related to oxygen binding and are not observed in the reduced minus oxidized spectrum (Figure 2) or in the derived spectrum of cytochrome *d* (Figure 4). The spectrum of cytochrome a_1 (Figure 4) is particularly interesting in that it is similar to the spectra of high-spin heme *a* model compounds (Carter & Palmer, 1982). It is also similar to the proposed spectrum of the cytochrome a_3 component of the mitochondrial cytochrome *c* oxidase (Vannest, 1966) and nearly identical with the spectrum reported for the *a*-type cytochrome of *Corynebacterium* 7E1C (Jurtshuk et al., 1975). The presence of a prominent β band at 562 nm is particularly interesting. This could be the source of complications in previous spectroscopic and electrochemical analyses of the *b*- and possibly *c*-type cytochromes in the *E. coli* membrane. The low-temperature (77 K) difference spectrum of the purified cytochrome *d* terminal oxidase complex shows a distinct shoulder near 550 nm which cannot be attributed to a *c*-type cytochrome (Miller & Gennis, 1983). Possibly this spectral component is the β band of cytochrome a_1.

Inspection of Figure 4 shows that the isolated spectral contribution of cytochrome a_1 to the complex is not insignificant compared to the spectra of cytochromes *b*-558 and *d*. Cytochrome a_1 is not an impurity in the preparation and probably is present stoichiometrically as an integral component of the complex. A considerable effort has been made to

identify the prosthetic group of cytochrome a_1. All attempts to extract heme *a* from the complex failed, including protocols which were successfully used to remove heme *a* from the beef heart mitochondrial cytochrome *c* oxidase. Thin-layer chromatography was used to identify both heme *d* and heme *b*, but no third heme component was observed to be extracted. A solvent system was used which separated samples of authentic hemes *a*, *b*, and *d*. Previous work by Miller & Gennis (1983) showed that following SDS–PAGE the individual subunits of the complex were not associated with any detectable heme. This makes it unlikely that the prosthetic group of cytochrome a_1 is covalently attached to the protein.

One explanation of these observations is that the prosthetic group of cytochrome a_1 is not heme *a* but, rather, heme *b* in a highly unusual environment. This postulate is attractive in that it also provides an explanation for the unexpectedly large amount of heme *b* which was extracted from the purified complex (Miller & Gennis, 1983). Further work will be directed at both determining the structure of the prosthetic groups and, through the use of spectrophotometrically monitored coulometry, determining the quantity of each cytochrome in the functional oxidase complex.

Added in Proof

It was recently determined that the reduced minus oxidized spectrum of cytochrome *c* peroxidase closely resembles that of cytochrome a_1, thus strengthening the argument that the prosthetic group of cytochrome a_1 is actually heme *b*.

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Registry No. Cytochrome *d*, 9035-36-3; cytochrome *b*-558, 9064-78-2; cytochrome a_1, 9035-35-2; oxidase, 9035-73-8.

References

Barrett, J. (1956) *Biochem. J. 64*, 626.

Bragg, P. D. (1979) *Membrane Proteins and Energy Transduction* (Capaldi, R. A., Ed.) pp 341–449, Marcel Dekker, New York.

Carter, K., & Palmer, G. (1982) *J. Biol. Chem. 257*, 13507.

Castor, L. N., & Chance, B. (1959) *J. Biol. Chem. 234*, 1587.

Dutton, P. L. (1978) *Methods Enzymol. 54*, 411.

Edwards, C., Beer, S., Siviram, A., & Chance, B. (1981) *FEBS Lett. 128*, 205.

Goldberger, R., Smith, A. L., Tisdale, H., & Bomstein, R. (1961) *J. Biol. Chem. 236*, 2788.

Haddock, B. A., & Jones, C. W. (1977) *Bacteriol. Rev. 41*, 47.

Hendler, R. W., & Shrager, R. I. (1979) *J. Biol. Chem. 254*, 11288.

Hendler, R. W., Towne, D. W., & Shrager, R. I. (1975) *Biochim. Biophys. Acta 376*, 42.

Itagaki, E., & Hager, L. P. (1966) *J. Biol. Chem. 241*, 3687.

Jurtshuk, P., Jr., Mueller, T. J., & Acord, W. C. (1975) *CRC Crit. Rev. Microbiol. 3*, 399.

Kita, K., Kasahara, M., & Anraku, Y. (1982) *J. Biol. Chem. 257*, 7933.

Koland, J. G. (1983) Ph.D. Thesis, University of Illinois, Urbana, IL.

Koland, J. G., Miller, M. J., & Gennis, R. B. (1984) *Biochemistry 23*, 445–453.

Kranz, R. G., & Gennis, R. B. (1983) *J. Biol. Chem. 258*, 10614.

Lemberg, R., Stewart, M., & Bloomfield, B. (1955) *Aust. J. Exp. Biol. Med. Sci. 33*, 491.

Lorence, R. M., Green, G. N., & Gennis, R. B. (1984) *J. Bacteriol. 157*, 115–121.

Lowry, O. H., Rosenberg, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265.

Matsushita, K., Patel, L., Gennis, R. B., & Kaback, H. R. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 4889.

Miller, M. J., & Gennis, R. B. (1983) *J. Biol. Chem. 258*, 9159–9165.

Poole, R. K., Scott, R. I., & Chance, B. (1981) *J. Gen. Microbiol. 125*, 431.

Poole, R. K., Baines, B. S., Hubbard, J. A. M., Hughes, M. N., & Campbell, N. J. (1982) *FEBS Lett. 150*, 147.

Pudek, M. R., & Bragg, P. D. (1976a) *Arch. Biochem. Biophys. 174*, 546.

Pudek, M. R., & Bragg, P. D. (1976b) *FEBS Lett. 62*, 330.

Reid, G. A., & Ingledew, W. J. (1979) *Biochem. J. 182*, 465.

Rice, C. W., & Hempfling, W. P. (1978) *J. Bacteriol. 134*, 115.

Sawyer, D. F., & Roberts, J. L., Jr. (1974) *Experimental Electrochemistry for Chemists*, Wiley, New York.

Shrager, R. I., & Hendler, R. W. (1982) *Anal. Chem. 54*, 1147.

Spatz, L., & Strittmatter, P. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 1042.

Vannest, W. H. (1966) *Biochemistry 5*, 838.

Van Wielink, J. E., Oltmann, L. F., Leeuwerik, F. J., De Hallander, J. A., & Stouthamer, A. H. (1982) *Biochim. Biophys. Acta 681*, 177.

Wilson, G. S. (1978) *Methods Enzymol. 54*, 396.