Ed.) pp 138–146, Springer-Verlag, Berlin.
Lutz, M., Kléo, J., & Reiss-Husson, F. (1976) *Biochem. Biophys. Res. Commun. 69*, 711–717.
Lutz, M., Brown, J. S., & Rémy, R. (1979) in *Chlorophyll Organization and Energy Transfer in Photosynthesis* (Wolstenholme, G., & Fitzsimons, D. W., Eds.) pp 105–125, Excerpta Medica, Amsterdam.
Lutz, M., Hoff, A. J., & Bréhamet, L. (1982) *Biochim. Biophys. Acta 679*, 331–341.
Maggiora, G. M. (1979) *Int. J. Quantum Chem. 16*, 331–352.
Matthews, B. W., Fenna, R. E., Bolognesi, M. C., Schmid, M. F., & Olson, J. M. (1979) *J. Mol. Biol. 131*, 259–285.
Mc Elroy, J. D., Mauzerall, D. C., & Feher, G. (1974) *Biochim. Biophys. Acta 333*, 261–278.
Norris, J. R., Uphaus, R. A., Crespi, H. L., & Katz, J. J. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 625–628.
Paillotin, G., Vermeglio, A., & Breton, J. (1979) *Biochim. Biophys. Acta 545*, 249–264.
Parson, W. W. (1982) *Annu. Rev. Biophys. Bioeng. 11*, 57–80.
Parson, W. W., & Monger, T. G. (1976) *Brookhaven Symp. Biol. 28*, 196–212.
Parson, W. W., Scherz, A., & Warshel, A. (1985) in *Antennas and Reaction Centers of Photosynthetic Bacteria* (Michel-Beyerle, M. E., Ed.) pp 122–130, Springer-Verlag, Berlin.
Robert, B. (1983) Thèse de 3ème cycle, Université Pierre et Marie Curie, Paris.
Robert, B., & Lutz, M. (1985a) in *Spectroscopy of Biological Molecules* (Alix, A. J. P., Bernard, L., & Manfait, M., Eds.) pp 338–341, Wiley, Chichester, U.K.
Robert, B., & Lutz, M. (1985b) *Biochim. Biophys. Acta 807*, 10–23.
Robert, B., Szponarski, W., & Lutz, M. (1985) *Springer Proc. Phys. 4*, 220–224.
Schenck, C. C., Mathis, P., & Lutz, M. (1984) *Photochem. Photobiol. 39*, 407–417.
Serlin, R., Chow, H. C., & Strouse, C. E. (1975) *J. Am. Chem. Soc. 97*, 7237–7242.
Shipman, L. L., Cotton, T. M., Norris, J. R., & Katz, J. J. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 1791–1794.
Tronrud, D. E., Schmid, M. F., & Matthews, B. W. (1986) *J. Mol. Biol.* (in press).
Wasielewski, M. R., Smith, U. H., Cope, B. T., & Katz, J. J. (1977) *J. Am. Chem. Soc. 99*, 4172–4173.
Williams, J. C., Steiner, L. A., Feher, G., & Simon, M. I. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 7303–7307.
Zhou Qing (1985) Diplôme d'Etudes Approfondies, Université Pierre et Marie Curie, Paris.

Articles

Specific Overproduction and Purification of the Cytochrome b_{558} Component of the Cytochrome *d* Complex from *Escherichia coli*†

George N. Green, Robert M. Lorence, and Robert B. Gennis*
Departments of Biochemistry and Chemistry, University of Illinois, Urbana, Illinois 61801
Received April 16, 1985

ABSTRACT: In *Escherichia coli* strain GR84N[pNG10], the cloned gene for subunit I of the membrane-bound cytochrome *d* complex resulted in the overproduction of cytochrome b_{558} and facilitated purification of this cytochrome. Extracting membranes with 1% Triton X-100 followed by two chromatographic steps yielded a single band on sodium dodecyl sulfate–polyacrylamide gels corresponding to subunit I (M_r 57 000). Purified cytochrome b_{558} was in its native state as determined by difference absorption spectroscopy and by potentiometric analysis. Both the membranes of strain GR84N[pNG10] and the purified subunit I lacked the other two spectroscopically defined cytochromes, b_{595} (previously "a_1") and *d*, of the cytochrome *d* complex. Reconstitution of cytochrome b_{558} in phospholipid vesicles demonstrated that cytochrome b_{558} can be reduced by ubiquinol but that it does not reduce molecular oxygen. Heme extraction of cytochrome b_{558} yielded an extinction coefficient of 22 000 M^{-1} cm^{-1} for the wavelength pair of 560 and 580 nm in the reduced-minus-oxidized spectrum. The mutation on pNG10 that eliminates subunit II was mapped to a 250 base pair DNA fragment.

The cytochrome *d* complex is a two-subunit terminal oxidase found in the inner membrane of *Escherichia coli*. The purified complex contains three distinct cytochromes, cytochromes b_{558} and *d* and the cytochrome previously described as cytochrome a_1 (Miller & Gennis, 1983). Although cytochrome a_1 absorbs at 595 nm in the reduced-minus-oxidized spectrum, it does not contain an *a*-type heme (Miller & Gennis, 1983) but apparently contains protoheme IX (Lorence et al., 1986). Therefore, cytochrome a_1 will be referred to as cytochrome b_{595} in this report.

Cytochrome b_{595} has a second absorbance peak at 560 nm that overlaps with the absorbance of cytochrome b_{558} (Koland et al., 1984), an overlap that has made the quantitation of cytochrome b_{558} difficult. Subunit I has been previously shown (Green et al., 1984b) to be the cytochrome b_{558} component

†Supported by grants from the National Institutes of Health (HL16101) and the U.S. Department of Energy (DEA02-80ER10682). G.N.G. was supported in part as a National Institutes of Health Trainee under Grant GM07283. R.M.L. was supported in part by a University of Illinois fellowship.
* Correspondence should be addressed to this author at the Department of Chemistry.

0006-2960/86/0425-2309$01.50/0

of the complex, but attempts to separate the two subunits of the purified complex have resulted in denaturation and loss of heme (M. Miller, personal communication). In this report, an alternative method was used to obtain purified cytochrome b_{558} from a strain overproducing this protein. This strain was isolated as a mutant of the cloned *cyd* gene (Green et al., 1984a), the gene locus coding for both subunits of the complex. As a result of the mutation, subunit II is not synthesized although subunit I is made and inserted into the membrane. Strains carrying the mutated plasmid, pNG10, overproduce cytochrome b_{558} by 3–4-fold. This allows a simple purification protocol consisting of Triton X-100 solubilization of *E. coli* membranes and two DEAE column chromatography steps.

The purified protein appears similar to the membrane-bound form on the basis of spectroscopic and potentiometric studies. Heme extraction of cytochrome b_{558} yields an extinction coefficient of 22 000 M^{-1} cm^{-1} for the wavelength pair of 560 and 580 nm in the room temperature reduced-minus-oxidized spectrum. As expected from antibody inhibition studies (Kranz & Gennis, 1984), cytochrome b_{558} is reduced by ubiquinol, but it does not react with molecular oxygen. In the intact complex, the oxygen binding site is associated with heme *d*, on subunit II. Finally, the mutation that results in the loss of subunit II has been mapped to a 250 base pair *Bst*EII–*Cla*I DNA fragment that may contain part of the coding sequence for subunit II.

Materials and Methods

Materials. The detergents used were Triton X-100 (Amersham), sodium cholate (Sigma), sodium *N*-lauroylsarcosine (Sigma), Tween 20 (Sigma), and Zwittergent 3-12 (Calbiochem Behring). Calf intestinal phosphatase was obtained from Boehringer. The restriction enzymes and T4 DNA ligase were obtained from Bethesda Research Laboratories or New England Biolabs. Other chemicals were obtained from Sigma. Ubiquinone 1 was a gift of Hoffmann-La Roche.

Strain and Growth Condition. E. coli strain GR84N-[pNG10], *cydA*2, *nadA*1, *rpsL*, *recA*, was grown at 150 rpm in a 250-L fermentor at a sparge rate of 2 ft^3/min in a sodium DL-lactate minimal medium supplemented with nicotinic acid, casamino acids, and tetracycline (Tet)[1] (Green et al., 1984b). The antibiotic helps to maintain the plasmid pNG10. *E. coli* strains containing *cydA* mutations do not contain either subunit of the cytochrome *d* complex (Green et al., 1984b).

Plasmid Isolation. Plasmid pNG10 was isolated as a spontaneous mutant of plasmid pNG2 which contains wild-type *cyd* (Green et al., 1984a). The screening procedure involves a novel assay based on the color characteristic of strains containing cloned *cyd* (Green et al., 1984a). Overproduction of the cytochrome *d* complex, a green protein, imparts a yellow-green color to *E. coli* colonies on agar plates. Mutations in cloned *cyd* resulting in complete loss of color correspond to the loss of both subunits of the cytochrome *d* complex. The retention of color, but loss of a green tint, usually means that only subunit II is missing. The latter case applies to GR84N[pNG10]. The color retention is most apparent with the cell paste, which is pink for GR84N[pNG10] due to the overproduction of cytochrome b_{558}. By contrast, the cell paste for GR84N[pNG2] is dark green. The specific loss of one or both subunits was originally verified by immunoblotting following SDS–PAGE of membranes from these strains, using antibodies specific for either subunit I or subunit II of the complex (Green et al., 1984b).

Plasmid pNG6 was constructed by cutting pNG2 with *Cla*I followed by extractions with phenol and chloroform, then ethanol precipitation, and ligation of the dissolved DNA pellet with T4 DNA ligase (Maniatis et al., 1982). Plasmid DNA containing the deleted *Cla*I DNA fragment was detected among Tet-resistant transformants of strain GR84N by yellow colonies. Restriction analysis of a plasmid from one transformant indicated the plasmid was pNG6. GR84N[pNG6] overproduces subunit I, and cytochrome b_{558}, but makes no subunit II, a result similar to pNG10.

Purification of Cytochrome b_{558}. The purification was carried out at 4 °C. Approximately 30 g of frozen cells was suspended in 100 mL of buffer containing 200 mM Tris, 50 mM EDTA, and 1 mM phenylmethanesulfonyl fluoride (pH 8.5) by using an electric blender. Antifoam A spray (Dow Corning) was used to prevent foaming. The suspension was passed 3 times at 5 mL/min through a French pressure cell at 18 000 psi. Unbroken cells were pelleted by centrifugation for 20 min at 10000*g* in a Sorvall GSA rotor. The supernatant was then centrifuged at 200000*g* in a Beckman type 60 Ti rotor for 1 h. All centrifugation forces are specified for the bottom of the tubes. The pellet was solubilized in 50 mL of 50 mM potassium phosphate buffer, pH 8.0, containing 5 mM EDTA and 1% Triton X-100. A Ten Broeck tissue grinder was used to disperse the pellet. Following centrifugation at 200000*g* for 1 h, the supernatant was dialyzed for 4 h each against two changes of 1 L of 20 mM potassium phosphate buffer, pH 7.0, containing 1% sodium cholate and 2 mM EDTA, hereafter referred to as dialysis buffer. The supernatant was then loaded on a DEAE-Sepharose CL-6B (Pharmacia) column (15 × 1.5 cm) that was equilibrated with dialysis buffer, and the loaded sample was washed with 50 mL of dialysis buffer and eluted at 40 mL/h with an 800-mL gradient running from 0 to 1.2 M KCl in dialysis buffer. Fractions of 5 mL were collected. Those fractions with an Abs_{413}/Abs_{280} ratio greater than 0.2 and with a typical *b*-cytochrome absorbance in the visible region of the dithionite reduced minus ferricyanide oxidized difference spectrum were pooled and dialyzed for 2 h against two changes of 1 L each of dialysis buffer, adjusted to pH 7.5. The cytochrome sample was then loaded on a DEAE-Sepharose CL-6B column (15 × 2.5 cm) that was equilibrated with dialysis buffer (pH 7.5) and eluted at 40 mL/h with a 350-mL gradient running from 0 to 1.2 M KCl in the dialysis buffer (pH 7.5). The purified cytochrome b_{558} eluted as a red band. Colored fractions with dithionite reduced minus ferricyanide oxidized spectra that indicated the presence of native cytochrome b_{558}, judged from the spectral shape in the α-band near 560 nm, and that contained one protein band by SDS–PAGE were pooled, concentrated by ultrafiltration (Amicon XM-50 membrane), and dialyzed overnight against 1 L of dialysis buffer.

Formation of Phospholipid Vesicles Containing Cytochrome b_{558}. Proteoliposomes reconstituted with cytochrome b_{558} were formed by the detergent dialysis method (Racker, 1979) as described by Miller & Gennis (1983). The vesicles were made from a mixture of three parts by weight of *E. coli* phosphatidylethanolamine to one part by weight of egg phosphatidylglycerol. Both lipids were in chloroform–methanol solutions. The lipids were dried down under nitrogen and put under vacuum for several hours in order to remove all of the organic solvents. A lipid concentration of 5 mg/mL was obtained by adding buffer containing 100 mM potassium phosphate (pH 7.0) with 50 mM sodium cholate. The solution was sonicated at 4 °C with a probe-type sonicator until clear.

[1] Abbreviations: Tet, tetracycline; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; Abs, absorbance; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

Table I: Purification of Cytochrome b_{558}

step	total cytochrome[a] (nmol)	total protein (mg)	specific heme content (nmol of heme b/mg of protein)	yield (%)
(1) whole cells (30 g)	2200	4800	0.46	100
(2) membranes	714	366	2.04	34
(3) Triton X-100 solubilization	524	190	2.76	24
(4) first DEAE-Sepharose CL-6B pool	203	18	11.3	9
(5) second DEAE-Sepharose pool	44	3.4	13.1	2

[a] Computed by using $\Delta E_{560-580} = 22\,000\ M^{-1}\ cm^{-1}$. This value is obtained by extraction and measurement of protoheme IX as described in the text.

A total of 0.5 nmol of cytochrome b_{558} was added for every milligram of lipid in solution. The solution was dialyzed against three changes of 200 volumes each of buffer for 6–8 h. Similar reconstituted proteoliposomes were prepared with the intact purified cytochrome d complex (Miller & Gennis, 1983). The complex was quantitated by using $\Delta\epsilon_{628-607} = 7.4\ mM^{-1}\ cm^{-1}$ for cytochrome d within the complex.

Reduction of the Cytochrome by Ubiquinone 1. The reduction of cytochrome b_{558} by ubiquinol 1 was measured as follows. A total of 3.5 nmol of purified cytochrome b_{558} or 2.5 nmol of the purified cytochrome d complex in reconstituted phospholipid vesicles was added to 8.0 mL of buffer containing 50 mM potassium phosphate (pH 7.0) with 1.8% by weight glucose and 0.1 mg/mL glucose oxidase (Sigma). After argon was passed over the solution and the solution was stirred for 30 min at room temperature in an air-tight cuvette to remove all of the oxygen, the absorbance change at 560 nm was then observed upon addition of 12 mM dithioerythritol and 25 μM ubiquinone 1. Ubiquinol 1 is formed by the reduction of ubiquinone 1 with dithioerythritol.

Oxygen uptake measurements were made as described previously (Green & Gennis, 1983). Ubiquinone 1 (22 μM) was mixed with varying amounts of reconstituted lipid vesicles containing cytochrome b_{558} or the cytochrome d complex and then equilibrated with air at 37 °C. The reaction was initiated by addition of dithioerythritol (28 mM). Oxygen uptake by the complex was found to be linear with time (3 min) over the range of cytochrome concentrations used.

Other Methods. Pyridine hemochromogen and protein assays (Miller & Gennis, 1983), spectroscopic and electrochemical studies (Lorence et al., 1984a), restriction, ligation, transformation, acrylamide gel electrophoresis, and plasmid purification methods (Green et al., 1984a) have been described.

Results

Purification of Cytochrome b_{558}. A representative preparation of cytochrome b_{558} is summarized in Table I. A number of detergents were tested to solubilize cytochrome b_{558} from strain GR84N[pNG10], but optimal results were obtained with Triton X-100. However, the subsequent steps of the procedure were performed in sodium cholate since cytochrome b_{558} bound irreversibly to the DEAE-Sepharose CL-6B column in Triton X-100. A single DEAE column resulted in subunit I that was over 90% pure as judged by the specific heme content and by SDS–PAGE. The elution profile of this column is shown in Figure 1. Cytochrome b_{558}, monitored by the absorbance of 413 nm, eluted as three peaks with the second and third peaks containing less protein contamination. The third peak contained pure subunit I but more than half of the material was denatured (see inset to Figure 1, spectrum C). The region between peaks 2 and 3 was pooled because it contained the best material (see inset to Figure 1, spectra A and B). Eluting the pooled material through another DEAE column at a slightly higher pH gave an increase in specific heme content (Table I). SDS–PAGE analysis of this final

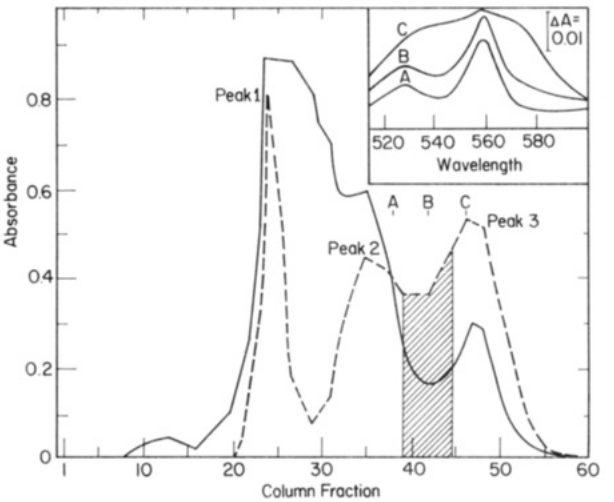


FIGURE 1: Elution profile of the first DEAE-Sepharose CL-6B column. Following the application of the sample (50 mL), the cytochrome was eluted by using a KCl gradient as described in the text. Peaks 1–3 eluted at KCl concentrations of 0.3, 0.5, and 0.75 M, respectively. Absorbance profiles shown are $Abs_{280} \times 0.1$ (—) and Abs_{413} (- - -). The inset shows dithionite reduced minus ferricyanide oxidized spectra of three column fractions (A–C) taken from peaks 2 and 3. The spectra demonstrate that peak 3 contained denatured cytochrome b_{558}. The pooled fractions are shown by the diagonal lines. The second DEAE column gave an Abs_{413} profile similar to that of the first DEAE column except that peak 1 was absent.

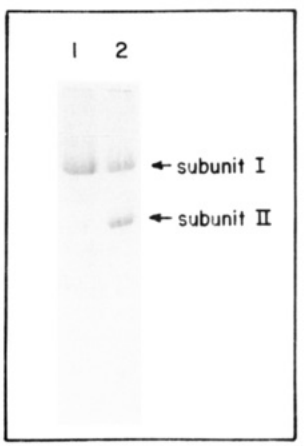


FIGURE 2: SDS–PAGE of the purified cytochrome b_{558} (lane 1) and the purified cytochrome d complex (lane 2).

product revealed a single protein band with the same mobility as subunit I of the pure cytochrome d complex (Figure 2). The pure protein was shown to be identical with subunit I by immunoblotting following SDS–PAGE and using monoclonal antibodies previously obtained (Kranz & Gennis, 1984) against subunit I of the cytochrome d complex (data not shown).

Potentiometric Analyses. Several lines of evidence show that the purified cytochrome b_{558} has similar properties to those of the cytochrome b_{558} part of the purified functional cytochrome d complex. In reconstituted phospholipid vesicles,

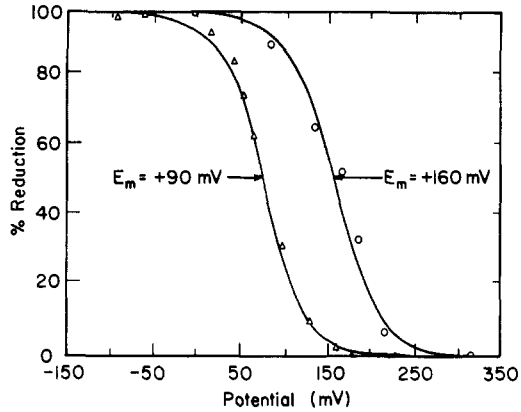


FIGURE 3: Potentiometric titrations of the purified cytochrome b_{558}. Titrations were performed in (Δ) 25 mM sodium cholate and 100 mM potassium phosphate, pH 7.0, and in (O) proteoliposomes in 100 mM potassium phosphate, pH 7.0. The formation and composition of the vesicles are described in the text. Percent reduction of cytochrome b_{558} was found by using Abs_{560} minus Abs_{577} of the dithionite reduced minus ferricyanide oxidized spectrum. Solid lines are the theoretical plots assuming a single one-electron component. The conditions for the chemical titration are described by Lorence et al. (1984a).

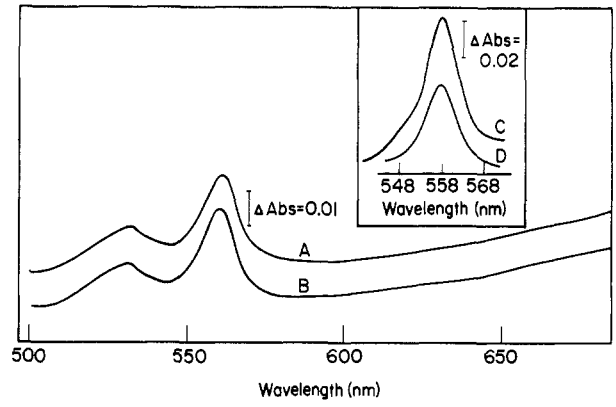


FIGURE 4: Dithionite reduced minus ferricyanide oxidized spectra of the purified cytochrome b_{558}. The room temperature spectrum of pure cytochrome b_{558} (A) (80 μg/mL) is compared to that of membranes of strain GR84N[pNG10] (B) (0.5 mg/mL). In the inset, the low-temperature reduced-minus-oxidized spectrum (77 K) of purified cytochrome b_{558} (D) (0.15 mg/mL) is compared to the purified cytochrome *d* complex (C) (0.25 mg/mL).

cytochrome b_{558} has an oxidation–reduction midpoint potential of about +160 mV (Figure 3). This value is the same value obtained for the cytochrome in the *E. coli* membrane, both in mutants lacking subunit II of the cytochrome *d* complex and in the wild-type strains containing the intact complex. Other mixtures of lipids were not tested with this reconstituted system.

The midpoint potential of pure cytochrome b_{558} was found to be sensitive to its environment. Solubilization in cholate results in a decrease in the midpoint potential to +90 mV (Figure 3). Similar effects of cholate solubilization were observed for the cytochrome b_{558} part of the cytochrome *d* complex (Lorence et al., 1984b).

Optical Spectroscopic Studies. The room temperature reduced-minus-oxidized difference spectrum of the purified cytochrome b_{558} was identical with that of the membrane of strain GR84N[pNG10] (Figure 4). This suggests that the purified cytochrome is not denatured. Low-temperature reduced-minus-oxidized difference spectroscopy was used to compare the spectroscopic properties of the purified cytochrome b_{558} and the cytochrome b_{558} portion of the purified cytochrome *d* complex. Both spectra (Figure 4) indicated identical peak maxima of the *b*-type cytochrome α-band at 558 nm. In contrast to the purified cytochrome *d* complex, purified cytochrome b_{558} had a low-temperature spectrum that lacked a shoulder at 548 nm. This result indicates that this shoulder is not attributable to asymmetry in the α-peak of cytochrome b_{558}, but rather it supports the hypothesis of Koland et al. (1984) that this spectral component is the β-band of cytochrome b_{595}.

The extinction coefficient of the pure cytochrome b_{558} was determined by using the pyridine hemochromogen technique. The spectrum of the hemochromogen (not shown) was typical of protoheme IX. The extinction coefficient of 22 000 M^{-1} cm^{-1} for the wavelength pair 560–580 was determined for the difference spectrum of purified cytochrome b_{558}. This value was the average of three extractions with a deviation of ±4%.

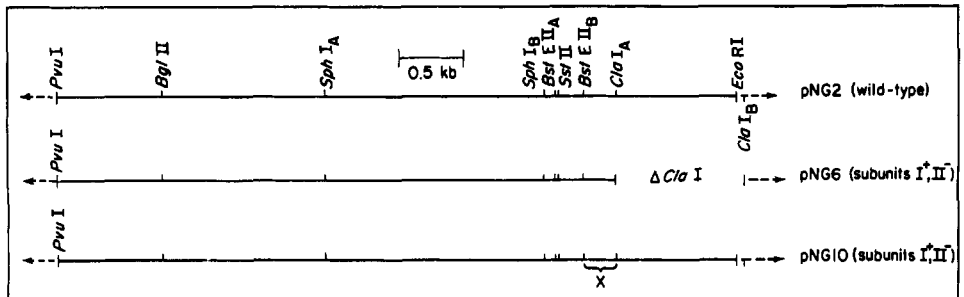
Reaction with Ubiquinol 1. Immunological studies (Kranz & Gennis, 1984) previously indicated that subunit I probably contains the site of ubiquinol oxidation. Cytochrome b_{558}, both in the purified form and as part of the cytochrome *d* complex, was reduced anaerobically by 25 mM ubiquinol 1 within seconds, although the pure cytochrome b_{558} was reduced at a slower rate. Dithioerythritol was used to generate ubiquinol 1 from ubiquinone 1. Some cytochrome b_{558} reduction by dithioerythritol was also observed in the absence of ubiquinol 1. Although cytochrome b_{558} was reduced by ubiquinol 1, the protein exhibited no oxygen uptake activity in the presence of ubiquinol even at high concentrations of cytochrome b_{558}. In contrast, the cytochrome *d* complex consumed oxygen at a rate of 360 μmol of O_2 min^{-1} (μmol of cytochrome *b*)$^{-1}$.

Mapping the Mutation within Cloned cyd. A restriction map of cloned *cyd* is shown in Figure 5. The location of the genes for subunits I and II within this cloned fragment has not been reported, but the results from pNG6 (Figure 5) indicate that subunit II may be encoded in part by the *Cla*I_A–*Eco*RI DNA fragment. However this region does not contain the mutation in pNG10, since the *Cla*I_A–*Cla*I_B restriction fragment from pNG10 restored a wild-type phenotype to pNG6 (Figure 5). Two other restriction fragments, *Bst*EII_A–*Bst*EII_B and *Sst*II–*Eco*RI, were analyzed. Only the *Sst*II–*Eco*RI fragment contained the mutation, indicating that this mutation lies between the *Bst*EII_B and *Cla*I_A restriction sites (Figure 5).

Discussion

Several detergents were tested for their ability to solubilize cytochrome b_{558} from the membrane. For example, sodium cholate and Tween 20 solubilized little of the cytochrome. Sodium *N*-lauroylsarcosine and Zwittergent 3-12 denatured cytochrome b_{558}. Zwittergent is used to solubilize the cytochrome *d* complex (Miller & Gennis, 1983), yet it denatures cytochrome b_{558} from strain GR84N[pNG10]. Apparently, subunit II of the complex, which is missing from this strain, protects subunit I from denaturation as it also protects subunit I from limited proteolysis (Green et al., 1984b). The extent of denaturation of cytochrome b_{558} was monitored spectroscopically after each of the two DEAE column chromatography steps of the purification. One peak eluting from these columns contained denatured cytochrome, possibly due to the high KCl concentration (>0.75 M), present in these fractions. However, attempts to renature the protein by dialysis in the absence of KCl were unsuccessful.

The best value for heme/protein for cytochrome b_{558} was 13.1 nmol/mg (Table I), with values ranging from 11.3 to 13.1 nmol/mg depending on the preparation. With the assumption of a 1:1 stoichiometry for heme to subunit I, and by use of



Restriction Fragment	Donor Plasmid	Recipient Plasmid	Phenotype
Cla I_A–*Cla* I_B	pNG10	pNG6	wild-type
Bst E II_A–*Bst* E II_B	pNG2	pNG10	subunits I^+,II^-
Sst II – *Eco* RI	pNG2	pNG10	wild-type

FIGURE 5: Location of the mutation of pNG10. For plasmids, pNG2, pNG6, and pNG10, (—) indicates cloned DNA that contains *cyd*, and (---) indicates the vector DNA derived from pBR322. (Δ) refers to deleted DNA, and (×) refers to the mutation on pNG10 that results in the loss of subunit II. I^+ and II^- indicates the presence and absence of subunits I and II, respectively, of the cytochrome *d* complex. A description of the mapping procedure for the mutation on pNG10 can best be described by using the *Bst*EII_A–*Bst*II_B restriction fragment. Plasmid pNG2 was cut with *Bst*EII yielding two DNA fragments. These fragments were separated on a 5% acrylamide gel and the smaller one was eluted from a crushed gel slice (Maniatis et al., 1982). Plasmid pNG10 was also cut with *Bst*EII and dephosphorylated. Purified *Bst*EII_A–*Bst*II_B fragment from pNG2 was added to the dephosphorylated pNG10 and ligated. An aliquot of dephosphorylated pNG10 was ligated without the DNA fragment as a control. The sample preparation yielded 205/210 yellow colonies, but 0/210 yellow-green (*cyd*$^+$) colonies, indicating the mutation on pNG10 was not located within the *Bst*EII restriction fragment. The five remaining colonies had lost their color and were not analyzed. The control yielded 79 yellow colonies. The yellow colonies from the sample preparation were found to contain, in about equal numbers, deletions and insertions of the *Bst*EII DNA fragment. Ten plasmids containing insertions were analyzed by restricting with both *SphI* and *Sst*II; the orientation of those inserts was identical with the orientation of the *Bst*EII DNA fragment originally found in pNG2. The high frequency of only one orientation may be due to nonidentical *Bst*EII restriction sites since one position in the site is degenerate.

the reported molecular weight for subunit I, 57 000 (Miller & Gennis, 1983), the pure protein should have a value of 17.5 nmol/mg. This difference may be due to the loss of heme, about 20%, from subunit I during the purification. It is interesting to note that when sonication was used to break the cells, a larger loss of heme was observed. The pure subunit isolated after this step contained only about 5 nmol of heme *b*/mg of protein.

The pure preparation of cytochrome b_{558} appeared to be very similar to the cytochrome b_{558} part of the cytochrome *d* complex as judged by spectroscopic and potentiometric studies. One difference was the slower rate of reduction of pure cytochrome b_{558} by ubiquinol 1. This difference is not surprising since ubiquinol, a two-electron reductant, may be oxidized by the cytochrome b_{558} part of the cytochrome *d* complex by a mechanism involving other heme groups in the complex. For the pure cytochrome b_{558} this interaction between heme groups is not possible. The slower reduction of cytochrome b_{558} may be due to denaturation, though the spectroscopic and potentiometric studies indicate that the cytochrome is purified in its native state. It is worth noting that the pure cytochrome b_{558} can also be slowly reduced by dithioerythritol, whereas in the complex, this cytochrome is not reduced under the same conditions.

One of the important results from this work is the measurement of an extinction coefficient for cytochrome b_{558}. The value of 22 000 M^{-1} cm^{-1}, obtained in this study, is twice as high as the value of 10 800 M^{-1} cm^{-1} found for the *b*-type cytochrome(s) in the purified cytochrome *d* complex (Miller & Gennis, 1983). This difference is explained by the hypothesis of Koland et al. (1984) that the prosthetic group of cytochrome b_{595}, formerly cytochrome a_1, is actually protoheme IX. The spectrum of cytochrome b_{595} is similar to that of cytochrome *c* peroxidase, having an α-band at 595 nm and a β-band at 560 nm. Thus, the measured extinction coefficient for the iron protoporphyrin IX bound to the intact cytochrome complex is abnormally low due to the fact that the complex contains two *b*-type cytochromes, b_{595} and b_{558}, and cytochrome b_{595} has a spectrum typical of a high-spin rather than the usual low-spin heme. This point is discussed further in the following paper (Lorence et al., 1986) along with evidence indicating that there is one cytochrome b_{558} present per cytochrome *d* complex.

While the mutation on pNG10 was being mapped, it was observed that deletions of the *Bst*EII_A–*Bst*EII_B DNA fragment still produced subunit I, indicating this subunit is encoded on the *Pvu*I side of *Bst*EII_A. Subunit II was not made by plasmids containing the *Bst*EII deletion, so the coding region for subunit II may extend into this region of the DNA. From the molecular weight of subunit II, 43 000 (Miller & Gennis, 1983), approximately 1.3 kilobases of DNA are needed to encode this protein, consistent with the DNA between *Bst*EII_A and *Cla*I_A being within the coding region for subunit II.

Registry No. Cytochrome b_{558}, 9064-78-2; cytochrome *d*, 9035-36-3; oxidase, 9035-73-8.

REFERENCES

Green, G. N., & Gennis, R. B. (1983) *J. Bacteriol. 154*, 1269–1275.

Green, G. N., Kranz, J. E., & Gennis, R. B. (1984a) *Gene 32*, 99–106.

Green, G. N., Kranz, R. G., Lorence, R. M., & Gennis, R. B. (1984b) *J. Biol. Chem. 259*, 7994–7997.

Koland, J. G., Miller, M. J., & Gennis, R. B. (1984) *Biochemistry 23*, 1051–1056.

Kranz, R. G., & Gennis, R. B. (1984) *J. Biol. Chem. 259*, 7998–8003.

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

Lorence, R. M., Miller, M. J., Borochov, A., Faiman-Weinberg, R., & Gennis, R. B. (1984b) *Biochim. Biophys. Acta 790*, 148–153.

Lorence, R. M., Koland, J. G., & Gennis, R. B. (1986) *Biochemistry* (following paper in this issue).
Maniatis, T., Fritsch, E., & Sambrook, J. (1982) in *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Miller, M. J., & Gennis, R. B. (1983) *J. Biol. Chem. 258*, 9159–9165.
Racker, E. (1979) *Methods Enzymol. 55*, 699–711.

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}†

Robert M. Lorence, John G. Koland,‡ and Robert B. Gennis*
Departments of Chemistry and Biochemistry, University of Illinois, Urbana, Illinois 61801
Received April 16, 1985

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

† Supported by Grant DEA02-80ER10682 from the U.S. Department of Energy and Grant HL16101 from the National Institutes of Health. R.M.L. was supported in part by a University of Illinois fellowship.
* Correspondence should be addressed to this author at the Department of Chemistry.
‡ Present address: Department of Chemistry, Baker Laboratory, Cornell University, Ithaca, NY 14853.

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

0006-2960/86/0425-2314$01.50/0