Isoelectric Focusing and Crossed Immunoelectrophoresis of Heme Proteins in the *Escherichia coli* Cytoplasmic Membrane

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Isoelectric focusing (IEF), agarose electrophoresis, and crossed immunoelectrophoresis (CIE) were used to resolve the heme-containing proteins of the *Escherichia coli* cytoplasmic membrane after solubilization by Triton X-100. Two bands in IEF stained for heme with pI values of 4.7 and 5.3. One of the bands, with an isoelectric point of pH 5.3, was present only when the cells were grown to late log or stationary phase and possessed N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD) oxidase activity. The pI 4.7 band was present in cells harvested in both mid-log and stationary phases. Agarose electrophoresis, using larger samples, revealed the same two components apparent by IEF, and, in addition, a third component. The heme-containing fractions were extracted after agarose electrophoresis and subjected to further study. The component which was present in cells grown to stationary phase contained hemes b, a1, and d. The other two fractions contained only b heme. One of these corresponded to the component with pI 4.7 in IEF and had catalase activity. Antisera were raised against Triton X-100-solubilized cytoplasmic membranes and against the focused TMPD oxidase complex. With these anti-sera, CIE in the presence of Triton X-100 revealed four precipitin complexes containing heme. Three of these corresponded to the components identified by IEF and agarose electrophoresis. We demonstrate that the combined use of IEF and CIE is valuable for analysis of membrane proteins. In particular, this work represents a substantial initial step toward a structural elucidation of the *E. coli* aerobic respiratory chain.

The cytochromes present in the *Escherichia coli* cytoplasmic membrane have been extensively studied by spectroscopic and potentiometric techniques. Shipp (34), using fourth-order finite-difference analysis of spectra, reported at least seven cytochromes, including two c cytochromes, three b cytochromes, cytochrome a1, and cytochrome d (previously a2). Subsequent workers have interpreted potentiometric titration data in terms of either two (29) or three (16, 17, 30) b cytochromes in addition to cytochromes a1 and d. Both cytochrome d and a b cytochrome, designated cytochrome o, bind to CO and are presumed to function as terminal oxidases (3, 30). There is conflicting evidence over the possible role of cytochrome a1 as a terminal oxidase (7, 11). A minimal scheme, certainly oversimplified, for the *E. coli* respiratory system appears to involve two parallel electron transport chains:

Given the uncertainty as to even the composition of the respiratory system, it is evident that such a scheme is useful only as a crude working model (for reviews, see references 12 and 18).

Little biochemistry has been attempted with the membrane-bound cytochrome components of the *E. coli* respiratory chain. Earlier workers reported some success at solubilization and initial attempts at biochemical resolution (1, 15). Kita and co-workers (20) reported the purification of the component referred to as cytochrome b556. Deeb and Hager (5), in earlier work, reported the purification of a b cytochrome (designated cytochrome b1) from *E. coli* W strain. The relationship between this cytochrome and those which have been spectroscopically characterized is unclear. In addition to strain differences, a further complication to biochemical studies is that the cytochrome composition of *E. coli* is highly dependent on the conditions under which
trolling the growth. However, by carefully controlling the growth conditions, the system can be greatly simplified for biochemical investigations (10, 28, 30, 32).

One purpose of this paper is to demonstrate the utility of both isoelectric focusing (IEF) and crossed immunoelectrophoresis (CIE) for the resolution and analysis of the *E. coli* cytochromes. Both techniques have been previously applied successfully to the study of membrane proteins (14, 26, 27, 36). In our study, four heme-containing protein complexes were resolved under nondenaturing conditions in the presence of Triton X-100. We show that one of the components, probably the cytochrome d terminal oxidase, has $N,N',N',N''$-tetramethyl-p-phenylenediamine (TMDP) oxidase activity. A second component contains only b heme and possesses catalase activity.

**MATERIALS AND METHODS**

**Chemicals.** Triton X-100 was obtained from Amer- sham Corp. TMDP was obtained from Marion Scientific Corp. as a 1% solution. 3,3',5',5'-Tetramethylbenzidine (TMBZ) was obtained from Sigma Chemical Co.

**Bacteria.** Most of the work was performed with an *E. coli* K-12 strain carrying the F152 episome (denoted F$_2$-gal in previous nomenclature). This strain, MR43L/F152, was obtained from W. Shipp and has been previously described (35). This strain was also used by Kita et al. for purifying cytochrome b$_{556}$ (20). The presence of the episome results in enhanced levels (two- or threefold) of apparently all of the cytochromes. Strain SHSP19 (33), which requires exogenous α-amino-levulinic acid (ALA) for heme biosynthesis, was made gal by mutagenesis with ethyl methane sulfonate. The F152 episome, which contains the gal operon, was then transferred into SHSP19 by the procedure described by Miller (25). The resulting strain, denoted ALA/F152, has two- to threefold-enhanced levels of all of the cytochromes and still requires exogenous ALA for heme biosynthesis.

**Growth conditions.** Cells were grown at 37°C on the basal medium of Cohen and Rickenberg (4) supplemented with 1 mM MgSO$_4$, 0.1 mM CaCl$_2$, 1.5 g of Casamino Acids (Difco Laboratories) per liter, and 5 g of D,1-sodium lactate per liter. The ALA/F152 strain was grown on glucose as the sole carbon source when ALA was not added.

Two types of growth conditions were used. Cells grown under conditions of high aeration in a 38-liter New Brunswick fermentor were harvested in early or mid-exponential growth phase. These cells have a pink color. The air flow rate was 25 liters/min, and the air agitation was 350 rpm. Cells grown under conditions of low aeration produce large amounts of cytochrome d, causing the cells to have a dark green color. The air flow rate for these cells was 5 liters/min, and agitation was at 200 rpm. These cells are harvested 5 to 10 h into stationary phase. The inoculum was a 100-ml culture grown overnight in Penassay broth.

**Absorption spectra.** Spectrophotometric measure- ments were made with an Aminco DW2 spectrophotometer. A small amount of sodium dithionite was used as the reductant, and potassium ferricyanide was used as the oxidant for reduced-minus-oxidized spectra.

**Preparations used for IEF.** Cytoplastic membranes of both mid-log and stationary-phase cells were isolated by the method of Yamato et al. (39). All IEF preparations used cytoplastic membranes as the starting point for solubilization. These membranes (approximately 4 mg of protein per ml) were solubilized by making the solution 1% Triton X-100, using a 10% Triton X-100 stock solution. The mixture was incubated for 30 min at room temperature. After a 1-h centrifugation at 90,000 × g at 5°C, the solubilized supernatant was used directly for IEF.

As an alternative method, the inner membranes were made 1% with sodium cholate, using a 20% sodium cholate stock solution, pH 7.2. The final protein concentration was approximately 4 mg/ml. This was saturated to 20% (NH$_4$)$_2$SO$_4$, using an 80% saturated (NH$_4$)$_2$SO$_4$ stock solution containing 0.05 M potassium phosphate, pH 7.0, and slowly stirred for 30 min on ice. After centrifugation at 78,000 × g for 2 h at 5°C, the supernatant was made 1% with Triton X-100, using a 10% Triton X-100 stock solution. This was dialyzed overnight at 5°C against 1,000 volumes of 0.2% Triton X-100, pH 7, and then centrifuged at 78,000 × g for one h at 5°C. The supernatant was used for IEF and had a final protein concentration of approximately 1 mg/ml. A comparison of both the spectral characteristics and potentiometric titration profiles of the intact membranes and the Triton X-100 extracts demonstrated that the cytochromes were similar in the two preparations (not shown). This indicated that the detergent was apparently not selectively removing specific cytochromes.

**IEF.** IEF was carried out by using an LKB multi-phor flatbed system. All focusing was done at 5°C in 2-mm-thick polyacrylamide gels. The gels contained 10% glycerol, 1% Triton X-100, and 5% acrylamide. A 3.5 to 9.5 pH gradient was made according to the manufacturer, using LKB ampholines. Gels were pre-focused at 300 V for 2 h. Application wells were filled with a 1% solution of amidosulfonic acid (approximately 1.5 ml cm$^{-2}$). The samples were applied in the wells and focused for approximately 1.5 h before more sample was applied to each well. Reapplication up to two times could be done. Focusing was carried out at 200 V for 8 to 10 h after the final sample application. The sample wells were cut near the cathode (approximately pH 8.0). Variations of gel composition, application methods, and focusing conditions were attempted, and the conditions described are critical.

**Preparations for CIE.** The following procedure was used for obtaining preparations to be used for CIE and agarose electrophoresis. Cells (5 g, wet weight) were suspended in 40 ml of 0.05 M potassium phosphate buffer—25% sucrose at 0.05 M EDTA (0.4 ml of a 1 M stock solution) and lysozyme (4 mg) were added, and cells were spheroplasted for 1 h at room temperature. After centrifugation at 11,000 × g for 30 min, the spheroplasts were suspended in 35 ml with 0.05 M potassium phosphate buffer, pH 7.2. Approximately 2 mg of DNase was added, and the samples were sonicated at 5°C for 26 min (50% pulse at 250 W). Whole cells were removed by centrifugation at 10,000
x g for 10 min. Membranes were pelleted at 160,000 x g in 2 to 4 h. The crude membrane pellet was homogenized in 8 ml of 0.05 M potassium phosphate buffer, pH 7.2. MgCl₂ was then added to a final concentration of 10 mM, using a 1 M stock solution. The membranes were solubilized by adding 8 ml of an 8% Triton X-100 solution and stirred slowly at room temperature for 30 min. After centrifugation at 90,000 x g for 1 h at 5°C, the supernatant was used for CIE. With radioactively labeled cells, the preparation was carried out as described above, but scaled down proportionately.

CIE. CIE was carried out in 1% agarose in a pH 8.6 Tris-barbital buffer system (obtained from LKB). Electrophoresis in the first dimension was for 3.5 h at 5 mA per plate, using 84 by 94-mm Gel Bond film (FMC Corp.). Rectangular sample application wells held approximately 60 µl of sample. After the first-dimension electrophoresis, plates were then sectioned if necessary to run only the proteins of interest against the selected antisera (as described below). Second-dimension electrophoresis was at 5 mA per plate (84 by 94 mm) and run overnight. Plates were pressed, washed in 0.1 M NaCl, and pressed again, each for 20 min. Heme staining was performed before, and protein staining was done after, plate drying.

Preparation of antiserum. Rabbits were injected with approximately 5 mg of inner membrane protein from stationary-phase cells solubilized in 4% Triton X-100. The preparation was emulsified with complete Freund adjuvant for the primary injection and with incomplete Freund adjuvant for monthly boosters. Sera were collected weekly after the first booster, and 500 ml of serum was treated according to Watt et al. (38) with sodium dextran sulfate, followed by ammonium sulfate precipitation. The ammonium sulfate-precipitated immunoglobins were redissolved in 1/10 the original serum volume. This was used for CIE and is referred to as the "anticytoplasmic membrane" serum.

For the "anti-TMPD oxidase" serum, a rabbit was immunized according to the following procedure. The P1 5.3 band in IEF which stained for both heme and TMPD oxidase activity was sliced out. Several samples were combined, and approximately 10 cm of the polycrylamide strips containing the TMPD oxidase was homogenized through a 20-gauge needle in a solution consisting of 1 ml of 1% Triton X-100 plus 1 ml of complete Freund adjuvant for the primary injection or 1 ml of incomplete Freund adjuvant for the monthly booster injections. The rabbit was bled weekly after the first booster, and 400 ml of the pooled serum was processed as above.

Heme, TMPD, and protein staining. Heme staining was carried out with TMBZ according to Thomas et al. (37). Protein staining was done with Coomassie brilliant blue. Staining with TMPD was performed by using a 1% TMPD solution obtained directly from Marion Scientific Corp.

Protein determinations. Protein determinations were carried out by using a modification of the method of Lowry et al. (21). All samples were made 1% with sodium dodecyl sulfate.

RESULTS

Figure 1 shows the reduced-minus-oxidized spectra of cells grown under conditions of high aeration (early log phase) and low aeration (stationary phase). These data are consistent with previous results from other laboratories (e.g., 30). Cells grown with high aeration and harvested at early log phase (approximately 3 mg of protein per ml). (B) Cells grown with low aeration and harvested in stationary phase (approximately 6 mg of protein per ml).
cessful IEF of these heme proteins, hemoglobin was used as a control. Using higher voltages or applying the samples toward the anode resulted in denaturation and loss of the heme. Even though the protein patterns were not altered, some heme-staining component, presumably free heme, was located close to the anode.

The protein pattern (Fig. 2B) for membranes from cells grown to stationary phase showed at least 12 major bands. The majority of the proteins were acidic, having pI values below pH 7.0. Figure 2A compares the heme-staining pattern for membranes from both log- and stationary-phase cells. Both preparations showed a heme-staining component with a pI of 4.7. This was the only heme-staining component present in membranes from mid-log, high-aeration cells. However, the stationary-phase membranes contained a single additional heme-staining component with a pI of 5.3.

Previous studies have shown that the membranes from cells grown under conditions of low aeration possess high TMPD oxidase activity (M. Miller and R. B. Gennis, unpublished data). The TMPD oxidase activity of the solubilized membranes from early-log-phase, high-aeration cells was less than 5% of the activity in solubilized membranes from stationary-phase cells. This activity was clearly associated with the heme-staining component, with pI = 5.3 (see Fig. 2C). The staining was done by directly applying the buffered TMPD solution to the top

![Image](http://jb.asm.org/)
of the IEF gel. Upon oxidation, the dye turned deep blue, and the activity was easily located.

Agarose electrophoresis in the presence of Triton X-100 could also be used to separate the heme-staining components in *E. coli* observed by IEF (Fig. 3). This corresponds to the first dimension of CIE. To verify that the TMPD oxidase activity and the heme-staining properties of the two components were dependent on the presence of heme, a mutant which requires exogenous ALA for heme biosynthesis was studied. This mutant, ALA/F152, was grown on glucose to stationary phase both with and without exogenous ALA. When ALA is omitted from the growth medium, no cytochromes were present by spectroscopic criteria (not shown), and the cells had no respiratory activity.

Two regions of the analytical agarose gel stained for heme when Triton X-100-solubilized stationary-phase membranes of strain MR43L/F152 were resolved electrophoretically (Fig. 3). The band with low mobility stained for TMPD oxidase activity. When these cells were grown on glucose instead of lactate, the TMPD complex appeared unchanged, but the amount of the second component appeared diminished (not shown). When membranes from strain ALA/F152 were grown in the presence of ALA on glucose, the TMPD complex was clearly present. However, growth in the absence of ALA resulted in the elimination of both the heme-staining and TMPD oxidase activities in the agarose gel. Clearly, heme was required for these activities.

The agarose electrophoresis was scaled up to a preparative level to permit further analysis. Samples containing as much as 30 mg of protein were loaded onto the preparative gel. The heme stain and TMPD oxidase stain revealed the same components observed by the analytical procedure. In addition, a third component, with intermediate mobility, was evident in samples prepared from early-log-phase cells. This component (b in Fig. 4) did not stain for heme, but was visible as a colored region in the preparative gel. Regions of the agarose gels were excised, and the proteins were extracted. Reduced-minus-oxidized difference spectra were recorded, and assays were made for TMPD oxidase and catalase. The heme-containing component with the lowest mobility present in stationary-phase cells contained hemes *b*, *a*₁, and *d* (Fig. 4a). In addition, this fraction possessed all of the TMPD oxidase activity in the membranes (Table 2). Although the electrophoresis resolved this component from other heme proteins, the specific activity did not indicate substantial purification.

The component in early-log-phase cells which did not stain for heme contained only *b* heme and did not have TMPD oxidase activity (Fig. 4; Table 2). This was the major heme protein in cells grown under conditions of high aeration to the early log phase. The component with fastest mobility, present in both log- and stationary-phase membranes, was a minor component, representing only about 5% of the total *b* heme present in log-phase cells (Fig. 4). It contained only *b* heme and, in addition, appeared to have high catalase activity. Solubilized *E. coli* cytoplasmic membranes possessed catalase activity (Table 2). The catalase activity was present in the membranes and not altered by solubilization in Triton X-100. It is reasonable to assume that the heme-containing component is responsible for this activity.

The *E. coli* K-12 strain we used has elevated cytochrome levels and was chosen to increase the chances of obtaining antibodies directed against these components. Antiserum was produced from rabbits challenged with solubilized cytoplasmic membranes from stationary-phase cells. CIE was performed with solubilized early-log and stationary-phase membranes, using this antiserum. Figure 5 shows the results with stationary-phase membranes. In both cases there appeared to be at least 50 precipitin arcs visible by Coomassie brilliant blue staining for protein. However, both early-log and stationary-phase membranes resulted in only one precipitin arc which stained for heme (Fig. 5c). This corre-
FIG. 4. Preparative agarose electrophoresis of Triton X-100-solubilized membrane proteins. (Top) Heme-stained electrophoretogram of stationary-phase cell preparation. Electrophoresis was performed as described in the text. The selected portions, (a), (b), and (c), were excised, eluted, and concentrated. (a) and (c) were from a stationary-phase cell preparation. (b) was from an early-log-phase cell preparation. (Bottom) Difference spectra of the eluates from (a), (b), and (c) recorded at room temperature. Proteins were eluted from the agarose by gently homogenizing the agarose sections in 0.05 M sodium phosphate, pH 7 buffer with 0.05% Triton X-100 in a volume of approximately five times the agarose volume. The proteins were allowed to elute overnight at 5°C, and then the agarose was removed by centrifugation at 60,000 × g for 1 h. The eluates were then concentrated with an Amicon apparatus, using an XM50 filter.

a precipitin arc corresponding to the TMPD oxidase when antiserum against stationary-phase membranes was used (Fig. 5a, d). Apparently, the component with pI 5.3 and with TMPD oxidase activity was not sufficiently antigenic in this preparation. Therefore, the region of the IEF gel containing the pI 5.3 component (the TMPD oxidase) was excised and used as an antigen to prepare antiserum directed against this material. The resulting antiserum showed a high titer against the TMPD oxidase and was able to precipitate up to 95% of the TMPD oxidase activity from preparations of stationary-phase membranes solubilized in Triton X-100 (data not shown). CIE studies using

| Table 2. TMPD oxidase and catalase activities in eluates from preparative agarose electrophoresis† |
|---------------------------------|-----------------|-----------------|-----------------|
| Prepn                          | Activity        | Heme b-specific content (nmol/mg of protein) |
| Triton X-100-solubilized       |                 |                 |                 |
| stationary phase                |                 |                 |                 |
| Eluate                          |                 |                 |                 |
| a                               | 2,340           | 0.05            | 2.1             |
| b                               | 2,770           | 0               | 2.4             |
| c                               | 0               | 0.77            | 0.05            |

† The eluates (see Fig. 4) were assayed for TMPD oxidase activity. Catalase activity was assayed according to Baudhuin et al. (2). The specific heme determination of eluate c required 30-fold concentration of the solution. The resulting high, unknown concentration of Triton X-100 may affect the protein determination in this case. The catalase assays did not require a substantial concentration of the eluate and were unaffected by the detergent.
this anti-TMPD oxidase serum and solubilized stationary-phase membranes showed that two of the major precipitin arcs stained for heme (Fig. 6). Of these, only one arc stained with TMPD. These two heme-staining precipitin arcs were absent from CIE patterns run with solubilized membranes which were isolated from cells harvested in early exponential growth (Fig. 6).

We also demonstrated that all three of the heme-staining precipitin arcs described contained heme. The ALA/F152 strain was grown in medium containing $[^3]HJALA$, and the presence of heme was detected on the CIE plates by autoradiography. Figure 7 shows the two heme arcs which were observed after using the anti-TMPD oxidase serum and solubilized stationary-phase membranes. These arcs, and no others, contained the radioactive label. Similarly, the precipitin arc corresponding to the pl 4.7 component also was shown to contain heme by this criterion, using anticytoplasmic membrane serum (not shown). In addition, another component, also very faint, was visible, corresponding to a component with a lower mobility in the first dimension than that of the pl 4.7 component. This was probably due to the presence of a low titer of antibodies directed against the cytochrome observed in agarose electrophoresis which did not stain for heme (Fig. 4b).

**DISCUSSION**

The *E. coli* aerobic respiratory chain is poorly characterized from a biochemical standpoint. Our purpose here was to demonstrate the analytical utility of IEF and CIE in resolving the heme-containing components in the *E. coli* cytoplasmic membrane. At this point, four components have been resolved in membranes solubilized in Triton X-100. Two of the heme-containing components are not present when the cells are grown under conditions of high aeration and are harvested at early-log growth phase. One of these components is a TMPD oxidase and is most likely the cytochrome $d$ terminal oxidase (31). These two components, which are resolved by CIE, are not split by IEF and are both contained in the band with pl 5.3.

One of the important conclusions of this work is the demonstration that a single heme-containing component is responsible for the observed TMPD oxidase activity in the membranes. This implies that TMPD is a selective electron donor to the cytochrome $d$-containing branch of the respiratory system. Hence, TMPD can be used to select for mutants deficient in cytochrome $d$, in a manner similar to that already reported for the selection of cytochrome-deficient mutants in other bacteria (e.g., 23, 24). A preliminary report of such work has already been published (G. N. Green, R. Faiman, and R. B. Gennis, Fed. Proc. 40:1669, 1981).

The heme-staining band with pl 4.7, which is present in both the log- and stationary-phase membranes, appears to be a single component. It corresponds to the only heme-staining precipitin arc which is present in CIE using serum directed against cytoplasmic membranes. The identity of this component is not known. It is a relatively minor heme-containing protein and is apparently responsible for the catalase activity of *E. coli* membranes. Experiments to test its function are in progress.

It is important to note that although the heme-containing protein with catalase activity is by spectroscopic analysis only a minor component,
FIG. 6. CIE of Triton X-100-solubilized membrane proteins from stationary- and early-log-phase cells, using anti-TMPD oxidase serum (as described in the text). These were stained with TMBZ, TMPD, and Coomassie brilliant blue. The TMPD oxidase staining was performed by adding a few drops of a 1% TMPD solution over the CIE plate and then pressing the plate. Approximately 250 μg of membrane protein was loaded and 400 μl of prepared antiserum per large (84 by 94 mm) CIE plate. (A) Heme stain; (B) TMPD oxidase stain; (C) Protein stain. SC, Stationary-phase cells; LC, log-phase cells.
it stains very heavily with the heme-specific TMBZ stain. In contrast, a major b-type cytochrome, present at a much higher concentration in early-log-phase cells than the "catalase," does not stain for heme in this procedure. The TMBZ stain is based on peroxidase activity and, clearly, heme proteins differ widely in their efficiency in catalyzing this reaction. On the other hand, the only proteins which did stain with TMBZ were shown to, in fact, contain heme, using the ALA/F152 strain and [3H]ALA for detecting these heme proteins.

In addition to staining the CIE plates for heme, zymograms were also made for the most common dehydrogenases, as previously reported (27, 36). Precipitin arcs were identified which contained residual D-lactate, succinate, and NADH dehydrogenase activities. In no case did these correspond to the major arcs containing heme. This is interesting in view of the recent reports that in Bacillus subtilis, succinate dehydrogenase, solubilized in Triton X-100, is associated with a b-type cytochrome (14). In beef heart mitochondria, the succinate-ubiquinone oxidoreductase (complex II) is also associated with a unique b cytochrome (13). This may not be the case for any of the common dehydrogenases in aerobically grown E. coli. Further work is being directed at verifying this point. For anaerobically grown E. coli, both nitrate reductase (22) and formate dehydrogenase (8, 9) may be weakly associated with b cytochromes.

IEF gels can also be stained for enzymatic activities. Succinate dehydrogenase was clearly focused and was not associated with either of the heme-staining bands in the gel. The same focusing protocol has been applied successfully to Rhodospseudomonas sphaeroides cytoplasmic membranes, as well as to purified E. coli D-lactate dehydrogenase and bacteriorhodopsin from Halobacterium halobium. The use of IEF, given specific staining and detection procedures, is of clear general value for studying membrane proteins and can be especially useful for producing high-titer antisera toward specific proteins (6).

One of the goals of this research is the production of monospecific antibodies directed toward each of the heme proteins in the E. coli cytoplasmic membrane. The use of both IEF and CIE methods in achieving this goal is essential. Such a library of antisera will be of great value, especially in conjunction with preparative biochemical methods and genetics, in deciphering both the structure and function of the E. coli aerobic respiratory chain.

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LITERATURE CITED


