

Immunological Analysis of the Heme Proteins Present in Aerobically Grown *Escherichia coli*

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Received 15 December 1983/Accepted 13 March 1984

Immunological methods were used to obtain information about *Escherichia coli* heme proteins. There is a membrane-bound catalase which consists of a single subunit (as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoretic analysis) which is also present in the soluble fraction. Antibodies raised against purified, soluble cytochrome b_{562} showed that this cytochrome is not related to any of the membrane-bound cytochromes, including the b_{562} component of the cytochrome *o* complex. Cytochrome b_{556} is immunologically unrelated to the cytochrome b_{556}^{NR} associated with the nitrate reductase system. Cytochrome b_{556} and cytochrome *o* are not present in a constant ratio in the membrane.

A number of heme-containing proteins have been identified in aerobically grown *Escherichia coli*. Most of these are *b* cytochromes. Four membrane-bound cytochromes have been purified. These are the cytochrome *d* terminal oxidase complex (15), the cytochrome *o* terminal oxidase complex (8, 11, 14), cytochrome b_{556} (9), and cytochrome b_1 (6). In addition, previous work from this laboratory indicated the presence of a membrane-bound heme protein with catalase activity (12). Several cytoplasmic heme proteins have also been isolated from *E. coli*, including cytochrome b_{562} (2, 13), two hydroperoxidases (4, 5), and a heme *b*-containing bacterioferritin (17, 18). The aerobic respiratory chain contains two branches (1). One consists of the cytochrome *d* complex, which functions as a ubiquinol-oxygen oxidoreductase (9a). The second branch terminates with the cytochrome *o* complex and is thought to require cytochrome b_{556} as an electron carrier (7). During the course of investigations on the aerobic respiratory chain, antibodies were raised against several of the *E. coli* heme proteins (see references 10 and 12). In this paper, these antibodies are utilized to address several specific questions concerning the possible interrelationships among these various heme proteins.

Methods. Antiserum against the membrane-bound catalase was prepared by immunizing a rabbit with precipitin arcs from nine crossed immunoelectrophoresis (CIE) plates as previously described (12). Antiserum against cytochrome b_{562} was obtained by using the purified cytochrome as antigen, 500 μg for the primary injection and 100 μg for the monthly booster (see reference 12). Cytochrome b_{556} was partially purified to 6 nmol of heme *b* per mg of protein as described previously (9, 10). The crude cytochrome preparation was then run on a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) system in parallel with a sample of purified cytochrome b_{556} . The portion of the gel containing proteins which comigrated with pure cytochrome b_{556} was excised and the proteins were eluted and cross-linked with glutaraldehyde. Approximately 100 μg of protein with incomplete Freund adjuvant was injected. For the booster, 20 μg of pure cytochrome b_{556} was cross-linked to keyhole limpet hemocyanin (2 mg) with glutaraldehyde

and injected with incomplete Freund adjuvant. Insufficient amounts of the purified cytochrome b_{556} were available to use as the primary injection. Rabbits were bled after 10 days.

CIE was performed with the antibodies against the catalase as described previously (12). Crude *E. coli* membranes (300 μg of protein) from strain MR43L/F152 (7) were solubilized in Triton X-100 and electrophoresed against 200 μl of antiserum. The subunit analysis of the catalase was performed by growing the cells in the presence of ^{35}S (see reference 11). The precipitin arc was excised, and the protein was analyzed by SDS-PAGE and autoradiography. Rocket immunoelectrophoresis was used to quantitate the levels of catalase after the cell suspensions were disrupted by sonication and the membranes were centrifuged and solubilized as previously described (1).

Rocket immunoelectrophoresis was also used to quantitate the levels of the cytochrome *o* and *d* complexes as in reference 11. The amount of cytochrome b_{556} was determined spectrophotometrically after immunoprecipitation of the cytochrome *o* and *d* complexes. Dithionite-reduced minus air-oxidized spectra were recorded, and the amount of *b* cytochrome was estimated by using the extinction coefficient $17,500 \text{ M}^{-1} \text{ cm}^{-1}$ for the wavelength pair 561 nm and 575 nm.

Membrane-bound catalase. CIE studies performed previously (12) revealed a heme protein which had catalase activity bound to the *E. coli* cytoplasmic membrane. This catalase is a minor heme protein in the membrane, probably accounting for less than 5% of the heme *b*. By using the precipitin arc as antigen, antiserum was raised against this protein. Results of experiments obtained with this antibody preparation are shown in Fig. 1. Figures 1A and B are CIE profiles of solubilized *E. coli* membranes run against this antiserum. Figure 1A shows a single rocket which stains for heme, and Fig. 1B shows that this corresponds to the major arc revealed by protein staining. It should be noted that the heme stain is basically a stain for peroxidase activity (16). Immunoprecipitation of solubilized *E. coli* membranes with this antiserum resulted in the precipitation of 100% of the catalase activity exhibited by the membranes (data not shown). Further analysis (SDS-PAGE) indicated that the protein precipitated in this arc consisted of only one subunit with a molecular weight of 70,000 (Fig. 1C). This information was obtained by growing cells in the presence of ^{35}S and excising the immunoprecipitated heme-containing arc, followed by analysis by SDS-PAGE and autoradiography.

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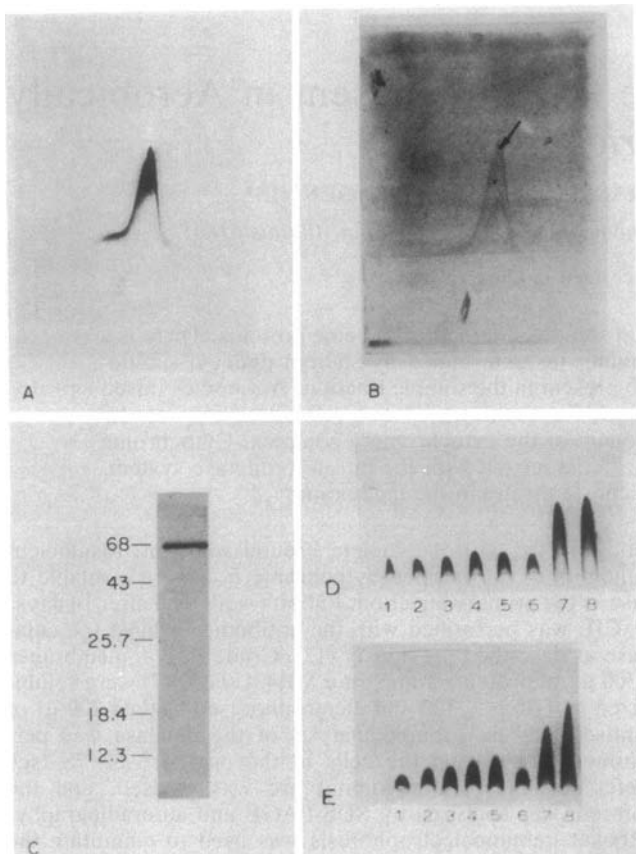


FIG. 1. Immunological analysis of the membrane-bound catalase. Details are provided in the text. (A) CIE plate stained for heme (16). (B) CIE plate stained for protein with Coomassie brilliant blue. Arrow indicates heme-staining arc. (C) Subunit analysis of the catalase. Molecular weights ($\times 10^3$) of standards are indicated on the left. (D) and (E) are rocket immunoelectrophoresis plates used to quantitate the amounts of the catalase in the soluble (D) and membrane (E) fractions from various cell preparations. Each plate contained 225 μ l of antiserum. The wells for the soluble fraction contained 94 μ g of protein, and the wells for the membrane samples contained 170 μ g of protein. Wells 1 to 5 are samples from cells grown on DL-lactate in a 28-liter fermentor which were removed at various times corresponding to the early exponential (well 1) to the stationary phase of growth (well 5). During cell growth, the oxygen tension was reduced by lowering the sparge rate after the first sample was collected (well 1). Wells 6, 7, and 8 are samples from small batch growths grown to stationary phase on glucose, DL-lactate, and succinate, respectively.

Rocket immunoelectrophoresis was performed with both soluble and membrane fractions of *E. coli* cells grown and harvested under a variety of conditions (Fig. 1D and E). It is clear that this heme protein is present in both the cytoplasm and the membranes. Cells grown on glucose had considerably less of this catalase, compared with cells grown on DL-lactate or succinate under identical conditions. It was also shown that this catalase is present in cells harvested in the exponential as well as in the stationary phase of growth.

Previous studies showed that the membrane-bound catalase has heme *b* as a prosthetic group (12). It was also shown that this enzyme manifests peroxidase activity, though this was not quantitated (12). The catalase and hydroperoxidase activity, along with the subunit molecular weight and the presence of heme *b*, suggest that this enzyme may be the same as hydroperoxidase I, purified as a soluble enzyme by Claiborne and Fridovich (4). The fact that a soluble catalase

is also found in the membrane may represent an *in vitro* artifact or may be of physiological significance. In any event, it is clear that a soluble catalase in *E. coli*, possibly hydroperoxidase I (4), is identical to the membrane-bound catalase. It is of interest to note the recent speculation that bacterioferritin, a soluble heme-containing protein in *E. coli*, may be identical to cytochrome *b*₁, previously isolated as a membrane-bound species (17).

Relationship between the soluble cytochrome *b*₅₆₂ and other *E. coli* cytochromes. Cytochrome *b*₅₆₂ is a small, soluble cytochrome from *E. coli* which has been extensively studied (2, 13) but whose physiological function is not known. There is also a cytochrome *b*₅₆₂ identified as a membrane component which has been recently shown to be part of the cytochrome *o* terminal oxidase complex (8, 11, 14). This complex contains four polypeptides, the smallest of which is the same size as the soluble cytochrome *b*₅₆₂ by SDS-PAGE analysis (11, 14). Antibody was prepared against the purified, soluble cytochrome *b*₅₆₂ (obtained as a gift from F. Scott Mathews). This antibody was used in immunoblotting experiments to test for cross-reactivity with various membrane-bound cytochromes, including the cytochrome *o* complex, the cytochrome *d* complex, cytochrome *b*₅₅₆, and cytochrome *b*₅₅₆^{NR}, the heme protein associated with *E. coli* nitrate reductase (3). Cytochrome *o* was obtained from an immunoprecipitin arc from a CIE plate (see reference 11). The cytochrome *d* complex was purified by M. J. Miller. Cytochrome *b*₅₅₆ was obtained as a gift from Y. Anraku, and cytochrome *b*₅₅₆^{NR} was the kind gift of J. DeMoss. The results clearly showed no cross-reactivity. Representative data shown in Fig. 2 (lanes 1 and 2) demonstrate that the soluble cytochrome *b*₅₆₂ is not derived, either intact or as a proteolytic fragment, from a membrane-bound *E. coli* cytochrome. This cytochrome must be considered to be a unique species, unrelated to the aerobic respiratory chain.

Relationship between cytochrome *b*₅₅₆ and other *E. coli* cytochromes. Similar experiments as described above for cytochrome *b*₅₆₂ were also carried out with cytochrome *b*₅₅₆.

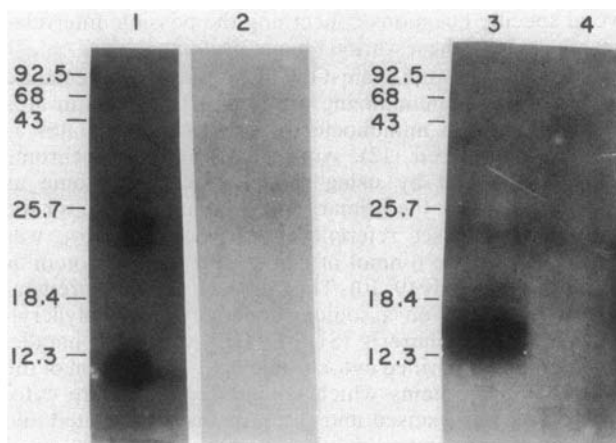


FIG. 2. Representative SDS-PAGE immunoblot analyses with antisera toward cytochrome *b*₅₆₂ and cytochrome *b*₅₅₆. Lanes 1 and 2 are immunoblots with anti-cytochrome *b*₅₆₂ (100 μ l per plate). Lane 1 contains 1 μ g of pure cytochrome *b*₅₆₂. Lane 2 contains 1 μ g of pure cytochrome *b*₅₅₆. Lanes 3 and 4 are immunoblots with antibodies against partially purified cytochrome *b*₅₅₆ (150 μ l per plate) and contained, respectively, 0.6 μ g of pure cytochrome *b*₅₅₆ and ca. 4 μ g of cytochrome *o* obtained from a CIE arc (see reference 11). Molecular weights ($\times 10^3$) of standards are shown to the left of lanes 1 and 3.

The cytochrome b_{556} was prepared from an *E. coli* mutant lacking cytochrome d which has been immunologically characterized (10). The procedure described by Kita et al. (9) yielded a spectroscopically pure cytochrome b_{556} . However, SDS-PAGE on this preparation showed multiple bands (data not shown). This partially purified preparation was used to raise antibodies, which were subsequently used to test for cross-reactivity. Y. Anraku kindly provided a 50- μ g sample of purified cytochrome b_{556} , and the antibody preparation did immunoblot with this sample. It was shown that cytochrome b_{556} is unrelated immunologically to the cytochrome o subunits, cytochrome b_{562} , the cytochrome d subunits, and cytochrome b_{556}^{NR} (Fig. 2, lanes 3 and 4). This is the first demonstration that the aerobic cytochrome b_{556} is immunologically distinct from the anaerobic cytochrome b_{556}^{NR} species.

Cytochrome b_{556} and cytochrome o are not coordinately regulated. Cytochromes o and b_{556} are thought to be parts of the high-aeration branch of the aerobic respiratory chain of *E. coli* (7). Earlier studies with the gel filtration procedure of Kita and Anraku (8, 9) showed that these are the major cytochrome components of the *E. coli* membrane in the absence of the cytochrome d complex and that they could be chromatographically resolved (10). Qualitatively, there have been indications from both chromatographic (10) as well as potentiometric (12a) methods that the ratio of cytochrome b_{556} /cytochrome o is not constant at different cell growth conditions. Immunological procedures can provide quantitative data to further explore these observations. By combining rocket immunoelectrophoresis data previously reported (11) and data from new immunoprecipitation experiments, the levels of cytochrome b_{556} , cytochrome o complex, and

cytochrome d complex have been determined. Cytochrome o complex and cytochrome d complex were quantitated through rocket immunoelectrophoresis by using monospecific antibodies against each. The antibodies raised against the partially purified cytochrome b_{556} were too polyspecific to be of direct use for quantitation. However, since cytochrome b_{556} is the only other major heme protein present in the membranes, it can be spectrophotometrically quantitated in solubilized membrane preparations after immunoprecipitation of both the cytochrome o and cytochrome d complexes. Figure 3 shows these cytochrome levels in samples taken from a single 28-liter batch growth of *E. coli*. After 9 h of initial growth, the oxygen flow rate was reduced from about 70 to 10% saturation. When the oxygen flow was reduced, the cytochrome d complex was apparently induced, whereas the concentration of cytochrome o drops, as reported previously (11). However, it appeared that cytochrome b_{556} remained at approximately the same concentration throughout this experiment. All of the data are normalized per milligram of membrane protein. The data show that cytochrome o and cytochrome b_{556} are not coordinately regulated. The significance of this observation will not be clear until the function of cytochrome b_{556} is known.

We would like to thank Y. Anraku, F. Scott Mathews, and J. DeMoss for kindly providing samples of purified *E. coli* cytochromes.

This study was supported by grant DEAC-02-80ER10682 from the Department of Energy and by Public Health Service grant HL16101 from the National Institutes of Health. C.A.B. was supported by a fellowship award from the Consejo Nacional de Investigaciones Científicas y Técnicas de la República Argentina.

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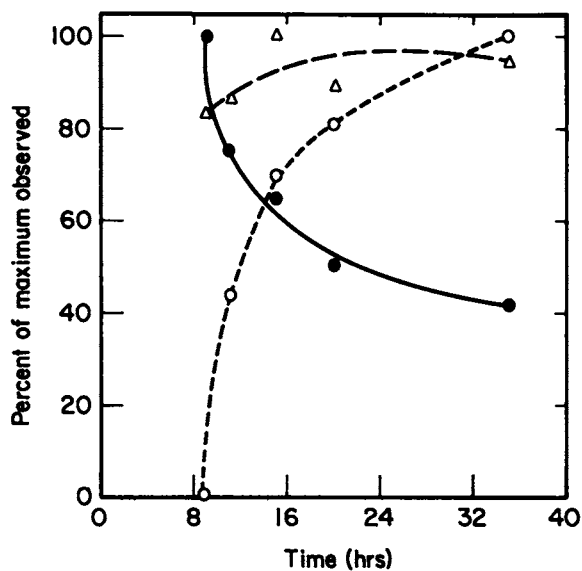


FIG. 3. Changes in the relative amounts of cytochromes o (●), d (○), and b_{556} (△) in response to a drop in oxygen tension during batch growth. The changes in the cytochrome o and d complexes were determined from rocket immunoelectrophoresis with monospecific antibodies. These data are replotted from Fig. 9 in reference 11 and are presented for comparison. The amount of cytochrome b_{556} was determined spectrophotometrically after immunoprecipitation of cytochromes o and d (see reference 11). The maximal level of cytochrome b_{556} was 0.5 nmol/mg of protein in the solubilized membrane preparations. The absolute levels of the cytochrome o and d complexes were not determined, and all these data are based on the relative heights of immunoprecipitation rockets.

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