The aerobic respiratory chain of *Escherichia coli* contains two terminal oxidases: the cytochrome d complex and the cytochrome a complex. Each of these enzymes catalyzes the oxidation of ubiquinol-8 within the cytoplasmic membrane and the reduction of molecular oxygen to water. Both oxidases are coupling sites in the respiratory chain; electron transfer from ubiquinol to oxygen results in the generation of a proton electrochemical potential difference across the membrane. The cytochrome d complex is a heterodimer (subunits I and II) that has three heme prosthetic groups. Previous studies characterized two monoclonal antibodies that bind to subunit I and specifically block the ability of the enzyme to oxidize ubiquinol. In this paper, the epitopes of both of these monoclonal antibodies have been mapped to within a single 11-amino acid stretch of subunit I. The epitope is located in a large hydrophilic loop between the fifth and sixth putative membrane-spanning segments. Binding experiments with these monoclonal antibodies show this polypeptide loop to be periplasmic. Such localization suggests that the loop may be close to His<sup>168</sup>, which has been identified as one of the axial ligands of cytochrome b<sub>558</sub>. Together, these data begin to define a functional domain in which ubiquinol is oxidized near the periplasmic surface of the membrane.

The cytochrome d complex is one of the terminal oxidases in the aerobic respiratory chain of *Escherichia coli* (see Ref. 1). Mutants of strains in which the cytochrome d complex is the only respiratory oxidase present in the membrane grow normally on nonfermentable substrates such as DL-lactate or succinate (2). The cytochrome d complex has been purified (3, 4) and demonstrated to catalyze the two-electron oxidation of ubiquinol-8 within the bilayer (5) and the four-electron reduction of molecular oxygen to water (6). Reconstitution studies have shown that electron flow through this enzyme from ubiquinol to oxygen is electrogenic and generates a proton motive force (5, 7). Hence, the cytochrome d complex is a coupling site in the respiratory electron chain.

The cytochrome d complex is a heterodimer, with one copy each of subunit I (58,000 Da) and subunit II (43,000 Da) (8). The enzyme contains three heme prosthetic groups: two protoheme IX and one heme d moieties (9-11). The two protoheme IX groups form two distinct cytochrome components: cytochrome b<sub>558</sub> and cytochrome b<sub>595</sub>. Cytochrome b<sub>558</sub> appears to be a six-coordinate cytochrome that is known to be located entirely within subunit I (12, 13). Cytochrome b<sub>595</sub> previously called cytochrome a<sub>1</sub>, appears to be a five-coordinate, high spin heme, and its function is not known (9-11, 14-16). It may play a direct role in the reduction of oxygen to water. Cytochrome d is definitely involved in oxygen binding (10, 17-20), and the heme d prosthetic group appears to be uniquely found in this enzyme (21).

Previous studies have strongly suggested that the catalytic active site at which ubiquinol oxidation occurs is spatially separate from the site at which oxygen is reduced (22). For example, trypsin proteolysis of the purified enzyme results in loss of ubiquinol oxidase activity but has no influence on the ability of the complex to oxidize the artificial electron donor $N,N,N',N'$-tetramethylphenylenediamine (22). Also, two monoclonal antibodies raised previously bind to the cytochrome d complex and inhibit ubiquinol oxidase activity without affecting the $N,N,N',N'$-tetramethylphenylenediamine oxidase activity of the enzyme (23). In each case, the target site was shown to be within subunit I.

The cyd operon, encoding both subunits of the enzyme, has been cloned (21) and sequenced (24). The deduced amino acid sequences suggest that both subunits I and II span the membrane, with seven and eight putative transmembrane segments, respectively (24). Some aspects of the topology of the subunits with respect to the bilayer have been elucidated using gene fusion techniques (25). The monoclonal antibodies that are the subject of this study identify a hydrophilic loop involved in quinol oxidation and localize it on the periplasmic side of the bilayer.

The topological evidence plus the measured stoichiometry of proton translocation catalyzed in the enzyme (H<sup>+</sup>/e<sup>-</sup> ≈ 1) (7) support a postulated model. It was proposed (22) that the enzyme functions by oxidizing ubiquinol at a site near the periplasmic surface, releasing two protons. The electrons are directed to a second site, located near the cytoplasmic surface, at which oxygen is reduced to water via a peroxy intermediate. The electron flow from the ubiquinol to the oxygen site generates a voltage across the membrane. The utilization of protons to form water on the cell interior and the release of protons as a result of ubiquinol oxidation near the periplasm are a scalar mechanism resulting in net proton flux.

The location of cytochrome b<sub>558</sub> (E<sub>m</sub> ≈ +180 mV) (9) on subunit I (12, 13) implicates this heme group in the oxidation of ubiquinol. This is consistent with the results of site-directed mutagenesis experiments in which His<sup>168</sup> within subunit I was altered to a leucine (26). The mutant complex specifically lacks cytochrome b<sub>558</sub> and is devoid of either ubiquinol or $N,N,N',N'$-tetramethylphenylenediamine oxidase activities.

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1 G. A. Newton and R. B. Gennis, manuscript in preparation.
His\(^{33}\) is probably located near the periplasmic surface of the membrane.

In the work described in this paper, the epitopes of the two inhibitory monoclonal antibodies have been mapped to the same 11 amino acid stretch within subunit I. These epitopes are located within a hydrophilic loop that is shown to be periplasmic and, therefore, very likely to be near to His\(^{33}\).

These data suggest the outlines of a domain within subunit I for the oxidation of ubiquinol.

**MATERIALS AND METHODS**

*Strains—* The tetracycline-sensitive *E. coli* strain GR84N, which lacks both subunits of the cytochrome d complex, was described previously (21). The *E. coli* strain Y1090 is a suppressor for the \(\lambda\) mutation S1, and its use as a host for Art1 has been described (29). The plasmid pNG2 contains the cyd gene and is used to overexpress the cytochrome d complex (21). The plasmid pFH101 also contains the cyd gene, but the vector allows for easier manipulation (26).

*cyd Fragment Library Construction in Art1—* Short random fragments of the cyd gene were produced from a plasmid containing the gene, pFH101 (26). 10 \(\mu\)g of DNA was digested with 1 ng of DpNase I in 100 \(\mu\)l of 20 mM Tris-HCl, pH 7.5, 1.5 mM MgCl\(_2\), 100 \(\mu\)g/ml bovine serum albumin for 60 min at 37 °C. The reaction was stopped by adding 200 \(\mu\)g/ml EDTA and applied to a Bio-Gel A-50 column (27). Three 25- \(\mu\)l fractions representing a range of fragments between approximately 400 and 100 base pairs were pooled and their DNA precipitated. The fragments were ligated to dephosphorylated Art1 arms (Promega Biotech) using EcoRI linkers (New England BioLabs) according to established protocols (29). The ligated DNA was packaged into phage heads using Gigapack (Stratagene), and the recombinant phage plated on *E. coli* Y1090. The library was screened according to Young et al. (29), using the monoclonal antibodies A14-5 and A16-1 (23) and an alkaline phosphatase-conjugated goat anti-mouse secondary antibody (Promega Biotech). Plaques from recombinant phage which expressed the monoclonal antibody epitopes were revealed by a purple signal upon incubating the nitrocellulose filters in an alkaline phosphatase substrate solution (300 \(\mu\)l nitroblue tetrazolium, 65 \(\mu\)g/ml 6-bromo-4-chloro-3-indolyl phosphate in 100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM MgCl\(_2\)). Several positive plaques for both A14-5 and A16-1 were isolated and purified.

*DNA Sequence Analysis of cyd Insert End Points in Art1—* Recombinant \(\lambda\) DNA was purified from plate lysates using LambdaScorb (Promega Biotech). Upon ethanol precipitation, the DNA was spooled out rather than recovered by centrifugation. The cyd insert end points in Art1 were determined by DNA sequence analysis using Sequenase (United States Biochemical) and the dideoxy chain-termination method (30). Two primers were used (New England BioLabs) which were complementary to the 5' and 3' sequence of the cyd gene on the plasmid site in Art1. Each was 24 nucleotides long. The "forward" primer consisted of the sequence GTGGGCGCAGCTCCGGGACCCG, and the "reverse" primer had the sequence TTGACACCGAGGACTGGTAATG. The annealing reaction consisted of approximately 100 ng of primer and 4 \(\mu\)l of X DNA. After heating to 70 °C for 2 min and cooling to 25 °C, the "labeling" reaction was performed according to the Sequenase protocol for 5 min at 25 °C using 1/10 dilution of the "labeling mix" and [\(\alpha\)-\(^{35}\)S]dATP. Termination reactions and denaturing gel electrophoresis were performed according to the Sequenase protocol. The DNA sequences of the Art1 inserts were correlated with the known cyd sequence (24) using DNASTAR (DNASTAR, Inc., Madison, WI).

**Labeling of Monoclonal Antibodies and Cytochrome d Complex**—The monoclonal antibodies A14-5 and A16-1 (23) were purified prior to labeling with an Affi-Gel protein A MAPS II column (Bio-Rad). The elution buffer was removed from the pooled fractions by repeated ultrafiltration (Amicon) using 100 mM potassium phosphate pH 7.2 as the dilution buffer. The antibodies were iodinated using Enzymobeads (Bio-Rad), sodium azide was added to 0.05% to stop the reaction, and unincorporated \(^{125}\)I was removed by Bio-Gel P-65G gel filtration in 100 mM potassium phosphate, pH 7.9. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the pooled fractions followed by autoradiography confirmed iodination of the monoclonal antibodies. Gamma counting and protein quantitation with Micro BCA protein assay (Pierce Chemical Co.) showed A14-5 and A16-1 to be labeled to 3600 and 4600 cpm/\(\mu\)g, respectively. Pure cytochrome d was labeled similarly, but 0.05% Sarkosyl, 500 mM NaCl, 10 mM Tris-HCl, pH 8.2, was used throughout. The reaction time was increased to 50 min due to decreased Enzymeactin activity in this buffer. The specific radioactivity was determined to be 2600 cpm/ng.

**Binding of Cytochrome d to Immulon Wells—* Cytochrome d complex was bound to Immulon wells (Dynatech) by adding it as a 5 \(\mu\)l volume in 0.05% Sarkosyl, 800 mM NaCl, 10 mM Tris-HCl, pH 8.2, and diluting to 50 \(\mu\)l with PBS (25, 26). The vesicles were incubated in such wells shown binding to be proportional to the amount of protein added and incubation. Greater than 30% binding was achieved over a 60-min incubation. The amount of bound antibody remained stable after three initial 5-min washes with PBS.

*Assay of A14-5 and A16-1 Binding to Cytochrome d Complex—* 50 \(\mu\)l of unlabeled cytochrome d complex was incubated in each well as described above for binding assays. After washing 3 \(\times\) with PBS, various dilutions of \(^{125}\)I-labeled monoclonal antibodies were added and incubated for 60 min. The wells were again washed 3 \(\times\) with PBS and counted. 50 ng of \(^{125}\)I-labeled cytochrome d complex was incubated in several wells and treated in parallel without any added antibody to serve as controls for the actual amount of cytochrome d complex bound.

**Inhibition of A14-5 Binding to Cytochrome d Complex by A16-1—* Cytochrome d was bound to Immulon wells as above and washed 3 \(\times\) with PBS. In each well, 600 ng of \(^{125}\)I-labeled A14-5 and various amounts of unlabeled A16-1 were incubated as 50-\(\mu\)l aliquots for 60 min. The wells were then washed 3 \(\times\) with PBS and counted.

*Preparation of Spheroplasts and Inside-out Vesicles—* For spheroplasts, GR84N and GR84N/pNG2 were grown to late log phase in Luria-Bertani broth and harvested by centrifugation at 10,000 \(\times\) g for 10 min. The cells were washed with 30 mM Tris-HCl, pH 8.0, pelleted, and 0.4 g wet weight, was resuspended in 10 ml of 20% sucrose, 30 mM Tris-HCl, pH 8.0. Phenylmethylsulfonyl fluoride was added to 200 \(\mu\)g/ml, EDTA was added to 10 mM, and lysozyme was added to 100 \(\mu\)g/ml. The cells were then incubated on ice for 30 min with occasional swirling. To decrease the viscosity of the solution due to DNA from some cell breakage, MgSO\(_4\) was added to 20 mM, and Dnase I was added to 50 \(\mu\)g/ml. After an additional 10 min on ice, the spheroplasts were collected by centrifugation at 10,000 \(\times\) g for 10 min and washed once with 20% sucrose, 30 mM Tris-HCl, pH 8.0, 200 \(\mu\)g/ml phenylmethylsulfonyl fluoride. The turbidity of the GR84N and GR84N/pNG2 spheroplast solutions as determined by absorbance at 600 nm was equalized by dilution with this buffer. Osmotic sensitivity was confirmed by dilution into deionized H\(_2\)O. Inside-out vesicles were prepared from GR84N/pNG2 as described previously (31), with the only modification being the addition of 300 \(\mu\)g/ml phenylmethylsulfonyl fluoride to all buffers. The amount of cytochrome d present in both vesicles and spheroplasts of GR84N/pNG2 was quantitated by difference spectra (10) and the vesicles diluted to achieve a cytochrome d concentration equal to that of the spheroplast size fraction.

**Binding of \(^{125}\)I A14-5 to Spheroplasts and Inside-out Vesicles**—Equal amounts of spheroplasts and vesicles were added to suitable tubes and centrifuged. The spheroplasts were spun down at 13,000 rpm in microcentrifuge tubes, and the vesicles were centrifuged for 20 min at approximately 110,000 \(\times\) g in a Beckman Airfuge using cellulose propionate Airfuge tubes (Beckman Instruments). The vesicle pellets were resuspended in 50 \(\mu\)l of PBS containing 10% fetal calf serum and 200 \(\mu\)g/ml phenylmethylsulfonyl fluoride. The spheroplast pellets were resuspended in the same buffer including 10% sucrose. After 60 min, the vesicles and spheroplasts were pelleted and resuspended in the same respective buffers containing 800 ng of \(^{125}\)I-labeled A14-5 and incubated for 60 min. The vesicle and spheroplast pellets were pelleted, washed 2 \(\times\) with their respective buffers, and counted.

**RESULTS**

The sequences within the cyd gene which encode the epitopes for the monoclonal antibodies A14-5 and A16-1 were mapped by constructing and screening a Art1 library containing small random fragments of the operon cyd. DNA fragments with random blunt end points were produced by digestion of pFH101 (26) with Dnase I in the presence of Mung bean nuclease. Fragments between 100 and 400 base pairs in size were inserted into Art1 arms, and the recombinant DNA was

\(^{2}\) The abbreviation used is: PBS, phosphate-buffered saline.
FIG. 1. A schematic map of the epitope for monoclonal antibodies A14-5 and A16-1. The horizontal bar at the top is a linear representation of cytochrome d complex subunit I where shaded areas are putative membrane-spanning regions. Corresponding horizontal lines below illustrate the extent of cyd DNA fragments inserted into λgt11 which encode polypeptides containing the epitope. Their exact end points in base pairs from the N terminus are on the right. The vertical lines indicate the extent of the epitope-encoding sequence as defined by the overlap of all positive clones. The amino acid sequence encoded by the overlapping region is shown at the bottom.

Lys-Leu-Ala-Ala-Ile-Glu-Ala-Glu-Trp-Glu-Thr

FIG. 2. The current topological model of subunit I of the cytochrome d complex. The epitope for the two inhibitory monoclonal antibodies is boxed on the large hydrophilic loop oriented toward the periplasmic side of the membrane. Also indicated is the location of His186, which is an axial ligand of cytochrome b56.
The DNA sequences that encode each epitope were determined using the dideoxy chain-termination method (Fig. 1). After masking, the wells were probed with a constant amount of $^{125}$I-labeled A14-5 sufficient to yield ~80% maximal binding combined with varying amounts of unlabeled A16-1. Percent inhibition represents the decrease in bound cpm at a given ratio of [A16-1] to [A14-5] relative to bound cpm with no A16-1 present.

Fig. 3. Inhibition of A14-5 binding to cytochrome d complex by A16-1. Pure cytochrome d complex was bound to Immulon wells as described under "Materials and Methods." After masking, the wells were probed with a constant amount of $^{125}$I-labeled A14-5. The actual amounts of antibody (mAb) bound were determined via prior specific radioactivity determination. The actual amount of cytochrome d complex bound was determined by performing the binding procedure in tandem using $^{125}$I-labeled cytochrome d complex of known specific radioactivity without adding labeled antibody.

Fig. 4. Binding curves for A14-5 and A16-1. Pure cytochrome d (cyt. d) complex was bound to Immulon wells as described under "Materials and Methods." After masking, the wells were probed with increasing concentrations of antibodies (Ab) A14-5 and A16-1. The actual amounts of antibody (mAb) bound were determined via prior specific radioactivity determination. The actual amount of cytochrome d complex bound was determined by performing the binding procedure in tandem using $^{125}$I-labeled cytochrome d complex of known specific radioactivity without adding labeled antibody.

The epitopes of the two inhibitory monoclonal antibodies have been clearly shown by this work to be within an 11-amino acid segment in subunit I. Some overlap of their sequence past the junction into the insert DNA. Sequences of sequences on either side of the EcoRI site were used to determine DNA insert end points were determined for 14 of the Xgtll monoclonal antibodies. Each revealed about 40 positive clones from approximately 5000 recombinant plaques screened. Of these, 20 plaques positive for each antibody were purified to subclones (Fig. 2).

DNA sequence common to them all was deduced and shown to be 33 base pairs long. The amino acid sequence shown in the context of a tentative topological model of subunit I in Fig. 2.

The location of the 11-amino acid stretch containing both epitopes is shown in the context of a tentative topological model of subunit I in Fig. 2.

The possibility of observing more counts with the spheroplasts over inside-out vesicles because doubts exist regarding the inside-out orientation prevents access by the monoclonal antibody to regions of the complex which are normally directed toward the periplasm. Values represent the mean of four samples, and the error bars equal 2 S.D.

Fig. 5. Binding of A14-5 to spheroplasts and inside-out vesicles. Preparation of spheroplasts and inside-out vesicles as well as binding of A14-5 monoclonal antibodies (mAb) to them is described under "Materials and Methods." In spheroplasts of E. coli strain GR84N containing the plasmid pNG2, which overproduces the cytochrome d complex (hatched bar), portions of the cytochrome d complex directed toward the periplasm are accessible to the monoclonal antibody A14-5. In spheroplasts of the cyd deletion strain GR84N (solid bar), no cytochrome d complex is present. In inside-out vesicles of GR84N/pNG2 (open bar), the cytochrome d complex is overexpressed, but the inside-out orientation prevents access by the monoclonal antibody to regions of the complex which are normally directed toward the periplasm.

DNA sequence common to them all was deduced and shown to be 33 base pairs long. The amino acid sequence shown in Fig. 1 was expressed in frame in each case.

The location of the 11-amino acid stretch containing both epitopes is shown in the context of a tentative topological model of subunit I in Fig. 2.

Since the two monoclonal antibodies bind within a single short amino acid stretch, competition for binding would be expected. Fig. 3 shows the inhibition of $^{125}$I-labeled A14-5 binding to cytochrome d complex by A16-1. Empirical constraints prevented determination of the A16-1 ratio at which maximal inhibition occurs. The appearance of inhibition after a 10-fold excess of A16-1 over A14-5 corresponds with the expected result quantifying binding to an equal amount of spheroplasts. Preparation of spheroplasts and inside-out vesicles as well as binding of A14-5 monoclonal antibodies (mAb) to them is described under "Materials and Methods." In spheroplasts of E. coli strain GR84N containing the plasmid pNG2, which overproduces the cytochrome d complex (hatched bar), portions of the cytochrome d complex directed toward the periplasm are accessible to the monoclonal antibody A14-5. In spheroplasts of the cyd deletion strain GR84N (solid bar), no cytochrome d complex is present. In inside-out vesicles of GR84N/pNG2 (open bar), the cytochrome d complex is overexpressed, but the inside-out orientation prevents access by the monoclonal antibody to regions of the complex which are normally directed toward the periplasm. Values represent the mean of four samples, and the error bars equal 2 S.D.

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DISCUSSION

The epitopes of the two inhibitory monoclonal antibodies have been clearly shown by this work to be within an 11-amino acid segment in subunit I. Some overlap of their sequence past the junction into the insert DNA. Sequences of sequences on either side of the EcoRI site were used to determine DNA insert end points were determined for 14 of the Xgtll monoclonal antibodies. Each revealed about 40 positive clones from approximately 5000 recombinant plaques screened. Of these, 20 plaques positive for each antibody were purified to subclones (Fig. 2).

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individual epitopes is probable since the ability of the monoclonal antibody A14-5 to bind to the cytochrome d complex is inhibited by the presence of A16-1. Although gross steric hindrance may be responsible for this observation, the short length of the 11-amino acid stretch sufficient to bind either monoclonal antibody suggests otherwise.

The mapping of the epitope within the primary sequence of subunit I has allowed determination of the position of an important hydrophilic loop with respect to the membrane bilayer. Through binding of the monoclonal antibodies to the cytochrome d complex in spheroplasts and vesicles, the large hydrophilic loop between the fifth and sixth putative membrane-spanning segments has been localized to the periplasmic side of the membrane. Because the monoclonal antibodies that bind to this loop specifically inhibit ubiquinol oxidase activity, this loop will be referred to as the “Q-loop.” Trypsin and chymotrypsin cleavage sites, which also cause specific inhibition of ubiquinol oxidase activity, have been mapped to the Q-loop within 30 amino acids of the monoclonal antibody epitope.3

The two-dimensional model of subunit I shown in Fig. 2 illustrates the location of the epitope in relation to the bilayer. It should be noted that the membrane-spanning regions shown are predicted from hydropathy profiles and therefore are not absolutely certain. However, it is evident that if the assignment of transmembrane segments is correct, the antibody-binding site lies on the same side of the membrane as His36. The strong possibility of close proximity between the epitope and His36 suggests that these residues are at or near the site at which ubiquinol is oxidized. Together, their location begins to define a functional domain for ubiquinol oxidation in the cytochrome d complex. At the very least, their location supports the postulate that ubiquinol is oxidized on the periplasmic side of the membrane and releases protons into the periplasm.

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