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Cytochrome pools in membranes of *Escherichia coli* grown aerobically on L-proline

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The cytochromes of membranes of the *cydA* mutant *Escherichia coli* GR19N grown on a proline-amino acid medium were examined. Reduced minus oxidized difference spectra (including fourth-order finite difference spectra) showed that cytochromes with absorption maxima at 554–555, 556–557, 560–561.5 and 563.5–564.5 nm were present. In addition, there were two components with absorption maxima at 548.5 and 551.5 nm which made a minor contribution to the α-band absorbance. These were not examined further. Two pools within the cytochromes were detected. One pool, which was reduced rapidly by the substrates NADH, formate and succinate, consisted of cytochromes of the cytochrome *o* complex. These cytochromes had absorption maxima at 555, 557 and 563.5 nm. In addition, the low-potential cytochrome associated with formate dehydrogenase was reduced rapidly by formate, and a component absorbing at 560–561.5 nm was also present in this pool. The second pool of cytochromes was reduced more slowly by substrate, although the rate was accelerated greatly in the presence of the electron mediator phenazine methosulfate. These cytochromes absorbed maximally at about 556.5 nm. A portion of the cytochrome in this pool was reoxidized by fumarate. This cytochrome may be a component of the fumarate reductase pathway, since the membranes showed high NADH-fumarate reductase activity. The respiratory chain inhibitor 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide appeared to act at two sites. One site of inhibition was between the dehydrogenases and the cytochromes. A second site of inhibition was located in the cytochrome *o* complex between cytochrome *b*-564 and oxygen.

Introduction

The respiratory chain of aerobically grown *Escherichia coli* has been the subject of many investigations (reviewed in Refs. 1–3). Our understanding of the organization of the respiratory chain has become clearer since the successful isolation of the two terminal respiratory chain oxidases [4–7]. Both complexes catalyze the reduction of oxygen with electrons derived from ubiquinol. The cytochrome *o* complex is responsible for the absorption maxima at 555 and 562 nm seen in reduced minus oxidized difference spectra measured at 77 K. Two mol heme/mol complex are present, but it is not clear which of the two hemes reacts with oxygen. In fact, the absence of distinct spectral changes produced by CO in the α-band region [3,8] raises some questions about

Abbreviations: DQH_2, duroquinol (2,3,5,6-tetramethyl-*p*-benzoquinol); Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HOQNO, 2-(*n*-heptyl)-4-hydroxyquinoline *N*-oxide; P-A, ascorbate in the presence of phenazine methosulfate; PMS, phenazine methosulfate.

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the identification of either *b*-555 or *b*-562 as cytochrome *o*. Cytochrome *o* is normally identified as the component showing a peak at 415 nm and a trough at 430 nm in reduced plus CO minus reduced difference spectra measured at 20 °C [9]. Under conditions of low aeration, the cytochrome *d* complex composed of cytochromes *b*-558, *b*-595 and *d* is formed. Cytochrome *d* appears to be the oxygen-reacting centre, since it forms an oxygenated intermediate [10].

Two other cytochromes have been purified from membranes of aerobically grown *E. coli*. Cytochrome *b*-556 has been purified as a soluble cytochrome in the presence of detergent [11]. It has a midpoint oxidation-reduction potential of −45 mV. Recently, its amino-terminal sequence has been found to be identical with that predicted from the DNA sequence of the *sdhC* gene of the succinate dehydrogenase operon [12]. The second cytochrome, cytochrome *b*-561, has a midpoint oxidation-reduction potential of +20 mV. It is a diheme cytochrome normally present at very low levels in membranes [13]. Its role, or association with a particular enzyme, is not presently known although Murakami et al. [12] have found that it can be reduced by D-lactate and NADH, and have suggested that it functions in transferring electrons between dehydrogenases and cytochrome *o*. A third cytochrome is found associated with formate dehydrogenase in cells grown anaerobically on nitrate. It has an absorption maximum at 555.5 nm and a midpoint oxidation-reduction potential of about −110 mV [14].

The preceding results suggest that the cytochromes of the aerobic respiratory pathways may be organized in two distinct groups. That is, moderate (+50 mV) to low (−120 mV) potential cytochromes associated with dehydrogenases and funneling electrons into the ubiquinone pool from which electrons are transferred to the terminal electron acceptor by the oxidase complexes. This mode of organization would thus be similar to that proposed for the formate dehydrogenase-nitrate reductase pathway [15,16].

This pattern of organization has been derived largely from studies of isolated complexes and cytochromes. Investigation of the behaviour of the cytochromes in situ in the membranes is complicated by the presence of numerous components, none of which appears to be resolvable by a single technique. The studies of Van Wielink [17,18], in which spectrum deconvolution is coupled with potentiometric analysis of membrane cytochromes of aerobically grown cells, have revealed four major components with α-band maxima at 555.7, 556.7, 558.6 and 563.5 nm, and with midpoint oxidation-reduction potentials of +46, +174, −75 and +187 mV, respectively. Although these components have not been ascribed to particular cytochromes, it is likely that the two components with the most positive potentials are associated with cytochrome *o*. There is less certainty about the identification of the other components. The −75 mV component could be the cytochrome of the formate dehydrogenase complex (E_m, −110 mV) [14]. Cytochrome *b*-556 of succinate dehydrogenase has a potential of −45 mV in purified preparations, but this value may be artifactually low due to modification by detergent [11]. Some workers have attributed the +46 mV component to cytochrome *b*-556 [19,20].

In the present paper we have used a number of techniques to study the cytochromes which were present in the membranes of *E. coli* grown aerobically on L-proline. The oxidation-reduction kinetics of the cytochromes were coupled with low-temperature difference spectroscopy to identify the presence of several kinetically distinguishable pools of cytochromes within the membranes. Although the use of the *cydA* strain GR19N reduced the complexity of the system to some extent by removing the contributions due to cytochromes *b*-558, *b*-595 and *d* [21], our results have indicated that the components of several respiratory chains are present together in the membranes. This increases the degree of complexity of the cytochrome composition above that recognized previously.

Materials and Methods

Bacterial strain, growth conditions and preparation of membranes. E. coli GR19N (F⁻ *thi*-1 *rha*-4 *lacZ*82 *gal*-33 *cydA*1) [21] was grown to the late exponential phase with vigorous aeration on a medium containing per liter: 7 g K_2HPO_4, 3 g KH_2PO_4, 0.5 g sodium citrate, 0.2 g $MgSO_4 \cdot 7H_2O$, 1 g $(NH_4)_2SO_4$, 5 g L-proline, 1.5 g casein

amino acids (Difco), 2.5 mg thiamine, 1 μmol ammonium molybdate, 1 μmol selenous acid and 12 μmol ferric citrate. The cells were harvested by centrifugation, washed twice with 0.9% (w/v) NaCl, and stored at −20°C.

Cells were suspended at 1.7 g/ml in 50 mM Hepes-KOH/5 mM magnesium acetate buffer (pH 7.4), and disrupted by passage twice through a French press at 1400 kg/cm^2. Unbroken cells were sedimented from the solution by centrifugation at 10000 × *g* for 20 min. The supernatant was centrifuged for 2 h at 252000 × *g* in a Beckman 60 Ti rotor. The sedimented membranes were washed by resuspension in Hepes/magnesium acetate buffer followed by centrifugation as before. The washed membranes were suspended for use at a protein concentration of 20 mg/ml in Hepes/magnesium acetate buffer.

Measurement of cytochrome reduction. The kinetics of reduction of the *b* cytochromes at room temperature were measured with a Perkin-Elmer 356 spectrophotometer operating in the dual-wavelength mode. The absorption of the sample at 559 nm was compared with the reference wavelength at 580 nm. The cuvette contained 0.5 ml membrane suspension, 1.5 ml Hepes/magnesium acetate buffer, 1 μl 3% H_2O_2, and any additions as noted in the legends to the figures. Reduction was initiated by the addition of substrate after a 3 min preincubation period. The presence of H_2O_2, which generated oxygen in the presence of endogenous catalase in the membrane preparation, ensured that the cytochromes were in their oxidized state at the commencement of the experiment.

Difference spectra at room temperature or at 77 K were measured with an SLM/Aminco DW2c double-beam dual-wavelength spectrophotometer. Nine spectra for each sample were collected and averaged. A slit width of 2.2 nm was used for the range 400–700 nm. For detailed examination of the alpha band region spectra were scanned between 515 nm and 590 nm using a slit width of 0.8 nm. Fourth-order finite difference spectra and the calculated difference between spectra were obtained by processing data with the Midan II kinetic processor/controller of the spectrophotometer.

Spectra at room temperature or 77 K were measured using the same buffer system as that used for the dual-wavelength kinetics studies. This allowed identification of the cytochromes which were reduced in the various steady-states observed by dual-wavelength spectrophotometry. Steady-states were trapped for spectroscopy either by removing samples without aeration to a precooled cryogenic cuvette or, for short periods of reaction, by carrying out the reaction at room temperature in the cryogenic cuvette. The reaction was then terminated by rapidly freezing the sample to 77 K.

Redox titration of the membrane cytochromes was carried out in 0.1 M potassium phosphate (pH 7.0) as described previously [22], except that the following mediators were present at a final concentration of 2 μg/ml: anthraquinone-2-sulfonate; 2-hydroxy-1,4-naphthoquinone; menadione; duroquinone; phenazine ethosulfate; phenazine methosulfate; 1,2-naphthoquinone; 2,6-dichlorophenolindophenol; hydroquinone; triphenanthroline cobalt (III); 1-monocarboxyl ferrocene. These mediators spanned the potential range of −225 to +530 mV.

Cytochrome o. Cytochrome *o* was purified by the method of Kita et al. [5] with the following modifications. The cytochromes were extracted from the membranes with Triton X-114. Chromatography on DEAE-Sepharose 6B was replaced by chromatography on DEAE-BioGel A. The purified cytochrome *o* showed two major bands (M_r, 55 and 32 kDa) and two weakly staining bands (M_r, 21 and 16 kDa) on sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by staining with 0.05% Coomassie blue in 10% (v/v) acetic acid and 25% 2-propanol. These bands correspond to those seen by Matsushita et al. [7]. A minor fifth band (M_r, 31 kDa) was also present.

Assays. Protein was measured by the method of Lowry et al. [23] using bovine serum albumin as a standard. Fumarate reductase activity was determined with benzyl viologen as described by Dickie and Weiner [24]. Oxidase activities were determined from the time required to deplete dissolved oxygen as indicated in dual-wavelength traces of the kinetics of reduction of the cytochromes (see above).

Results

At 77 K a dithionite-reduced minus oxidized difference spectrum of membranes of the *cydA* mutant, *E. coli* GR19N grown on a proline-amino acid medium showed an alpha-absorption peak at 556.5 nm with shoulders on the peak at about 547.5, 560 and 564 nm. There was a large peak at 428 nm. No absorption bands were present in the region 580–700 nm. This is consistent with the lack of the cytochrome *d* complex (cytochrome *b*-558-cytochrome *b*-595-cytochrome *d*) in this mutant. Cytochrome *o* was shown by a reduced plus CO minus reduced difference spectrum measured at room temperature. A peak at 415 nm and the trough at 430 nm demonstrated the presence of cytochrome *o* [1,9]. A distinct trough at 557–557.5 nm was present. At 77 K the spectrum showed in this region a minimum at 555 nm and a maximum at 566 nm. Similar spectra to those described above were observed in membranes from cells of GR19N grown on a glucose-salts medium.

Kinetics of reduction of cytochromes by substrate

Addition of substrate to a suspension of membranes leads to reduction of cytochromes. This was followed using dual-wavelength spectrophotometry by measuring the absorption change at 559 nm relative to the reference wavelength of 580 nm. As seen in Fig. 1, after an initial aerobic steady-state level of reduction, the major portion of the cytochrome became reduced when the dissolved oxygen in the buffer had been utilized. In all cases reduction occurred in two main phases – a 'fast' and a 'slow' phase, each of which was clearly not homogeneous. An inflexion between the phases was evident with formate, NADH and ascorbate (with PMS as an electron mediator). Under the conditions of the experiments, formate, NADH, succinate, ascorbate-PMS and duroquinol reduced 95, 86, 67, 55 and 38% of the dithionite-reducible cytochrome, respectively.

Reduced minus oxidized difference spectra with different substrates, and at different stages of reduction, are shown in Fig. 2. NADH (and formate, results not shown) gave a peak at 556.5 nm with shoulders at about 560 and 563.5 nm (curve 1). Succinate and ascorbate-PMS gave somewhat similar spectra, but with less cytochrome being

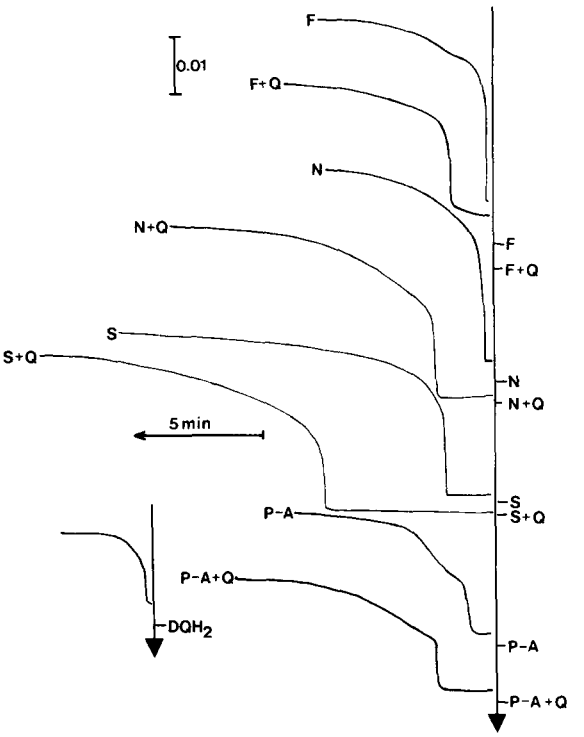


Fig. 1. Kinetics of reduction of cytochromes in membranes by different substrates in the absence and presence of HOQNO. Dual-wavelength measurements were carried out as described in Materials and Methods. The traces run from right to left. The vertical bar indicates absorbance units. Substrate was added at the arrow. F, 10 µl 1 M sodium formate; N, 50 µl 80 mM NADH; S, 10 µl 1 M sodium succinate; P-A, 15 µl 10' mM PMS followed by 25 µl 0.4 M potassium ascorbate; DQH_2, 40 µl 60 mM duroquinol; Q, 25 µl 10 mM HOQNO added 1 min before substrate.

reduced in the peak at 556.5 nm (curves 2 and 7). The NADH minus ascorbate-PMS difference spectrum (curve 3) demonstrated this more clearly, and also emphasized the complex nature of the peak at 556.5 nm. At least three components with absorption maxima at 554.5, 556.5 and 559 nm appeared to be present. Similar results were obtained with a NADH minus succinate difference spectrum (results not shown).

The slower kinetics of reduction of the cytochromes by ascorbate-PMS made it possible to investigate further the nature of the cytochromes reduced in the 'fast' and 'slow' phases. Reduction was allowed to proceed for a defined length of time in the cryogenic cell before the reaction was terminated by freezing to 77 K. As seen in Fig. 2,

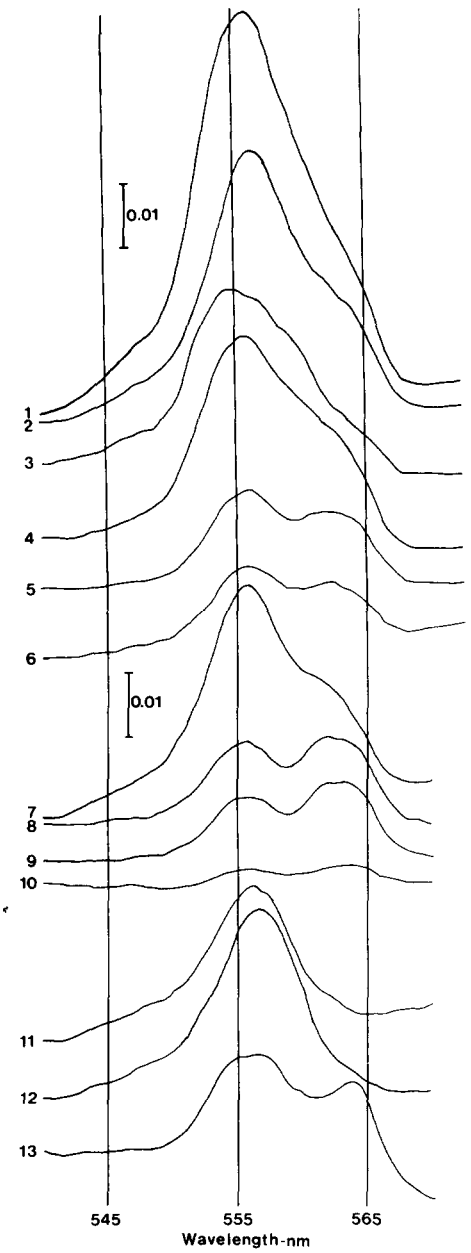


Fig. 2. Difference spectra of membrane cytochromes reduced by different substrates measured at 77 K. 1, NADH-reduced (for 15 min) minus H_2O_2-oxidized; 2, succinate-reduced (for 15 min) minus H_2O_2-oxidized; 3, calculated difference between spectra 1 and 7; 4, NADH-reduced (for 1 min) minus H_2O_2-oxidized; 5, NADH-reduced (for 0.5 min) minus H_2O_2-oxidized; 6, succinate-reduced (for 1 min) minus H_2O_2-oxidized; 7, ascorbate PMS-reduced (P-A reduced) (for 15 min) minus H_2O_2-oxidized; 8, P-A reduced (for 1.9 min) minus H_2O_2-oxidized; 9, P-A reduced (for 1.4 min) minus H_2O_2-oxidized; 10, P-A reduced (aerobic steady state) minus H_2O_2-oxidized; 11, calculated difference between spectra 7 and 8; 12, succinate-reduced (for 15 min) minus P-A reduced (for 1.9 min); 13, duroquinol-reduced (for 6 min) minus H_2O_2. The vertical bar indicates absorbance units.

curves 9 and 8, it was possible to terminate the reaction just at or after the end of the 'fast phase' of reaction (1.4 and 1.9 min). Two broad peaks of about equal size with maxima at 556 and 562.5 nm, but clearly showing heterogeneity, were evident. The 562.5 nm peak was reduced to a greater extent than the 556 nm peak in the sample taken at 1.4 min. Further reduction by ascorbate-PMS to a steady-state level at 15 min showed that the 'slow-phase' cytochrome had a broad absorption peak with a maximum at 556 nm (curve 7). The cytochromes reduced at the end of the 'fast phase' by NADH (curve 5) and by succinate (curve 6) had similar absorption spectra to those reduced by ascorbate (with PMS) (curves 9 and 8). Samples taken at slightly later time periods again showed that the increase in absorption was mainly in the 555–560 nm region (curves 4, 11 and 12).

The anaerobic state spectrum with duroquinol as reductant (curve 13) was similar to, but more clearly resolved than, that of the 'fast-phase' cytochrome reduced by ascorbate-PMS, NADH and succinate (curves 5, 6, 8 and 9). The alpha-absorption peak showed absorption maxima at 555, 557, 560.5 and 564 nm (Fig. 2, curve 13). With the exception of the peak at 560.5 nm, this spectrum is similar to that of purified cytochrome *o* (Fig. 3). The greater height of the absorption peak at 555–557 nm seen with membranes may be due to partial reduction of other cytochromes having absorption peaks at these wavelengths.

The alpha-absorption band in the reduced minus oxidized difference spectra appeared to be composed of at least four overlapping peaks. Support for this hypothesis was obtained by measuring fourth-order finite difference spectra of membranes reduced by different substrates (Fig. 4). Distinct separation of the peaks was not obtained in all derivative spectra although absorption maxima at 554–555, 556–557.5, 560–561.5 and 563.5–564.5 nm were seen. These corresponded to the peaks previously identified in the spectra shown in Fig. 2. There is some degree of variation in the positions of the maxima. This may be due

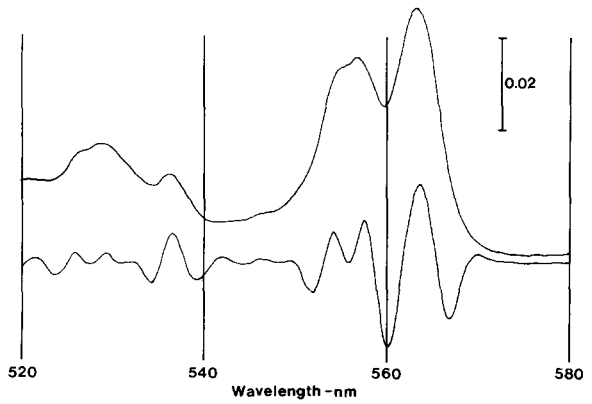


Fig. 3. Dithionite-reduced minus H_2O_2-oxidized difference spectra of purified cytochrome *o* complex measured at 77 K. The vertical bar indicates absorbance units. The lower curve is the fourth-order finite difference spectrum calculated from the upper curve.

to incomplete resolution of these peaks or possibly because more than one component contributed to a particular maximum. This will be discussed below. In addition, two peaks at 548.5 and 551.5 nm were present in the derivative spectra. These correspond to the shoulders seen on the short-wavelength side of the alpha peak in the original spectrum. These peaks were absent from the cytochrome *o* preparation, which showed maxima in the fourth order spectra at 554, 557.5 and 563.5 nm (Fig. 3).

Potentiometric titration of cytochromes in membranes

The midpoint oxidation-reduction potentials of the components constituting the alpha-band absorbance in the difference spectrum were determined by chemical titration in the presence of mediators as described in Materials and Methods. The data were analyzed by a non-linear least-squares fitting program [22]. The minimum number of components giving a satisfactory fit to the data-points was four. The midpoint potentials of these components, and their percentage contribution to the absorbance (in parenthesis), were −105 mV (13%), +8 mV (58%), +132 mV (15%) and +248 mV (14%) (Fig. 5). It is likely that some of these values represent the averaged potentials of more than one component rather than the value of a single cytochrome.

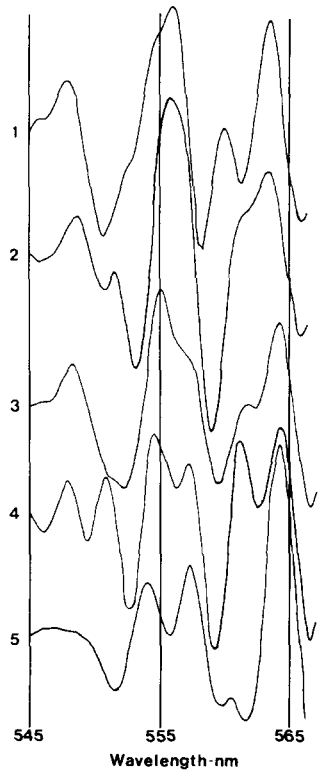


Fig. 4. Fourth-order finite difference spectra of membrane cytochromes reduced by different substrates measured at 77 K. 1, NADH-reduced (for 11 min) minus H_2O_2-oxidized; 2, succinate-reduced (for 12 min) minus H_2O_2-oxidized; 3, formate-reduced (for 3 min) minus H_2O_2-oxidized; 4, ascorbate-PMS-reduced (for 10 min) minus H_2O_2-oxidized; 5, duroquinol-reduced (for 6 min) minus H_2O_2-oxidized.

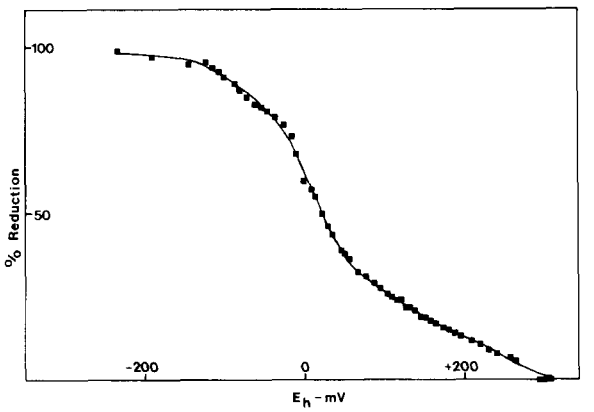


Fig. 5. Redox titration of cytochromes of membranes. The percentage reduction of the cytochromes is plotted against the applied oxidation-reduction potential (E_h). The line represents the theoretical curve drawn for four components with midpoint potentials of −105, +8, +132, and +248 mV and constituting 13, 58, 15 and 14% of the total absorbance, respectively.

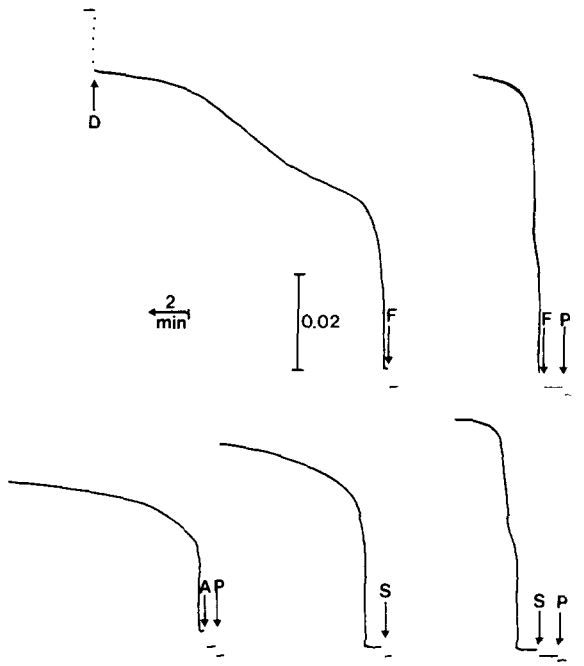
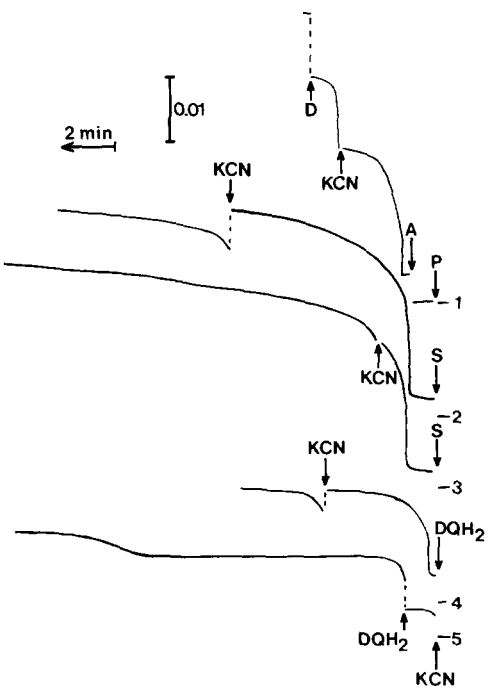


Fig. 6. Effect of PMS on the kinetics of reduction of the membrane cytochromes. Dual-wavelength measurements were carried out as described in Materials and Methods. The traces run from right to left. The vertical bar indicates absorbance units. Substrates were added where indicated. F, 10 µl 1 M sodium formate; P, 15 µl 10 mM PMS; S, 10 µl 1 M sodium succinate; A, 25 µl 0.4 M potassium ascorbate.



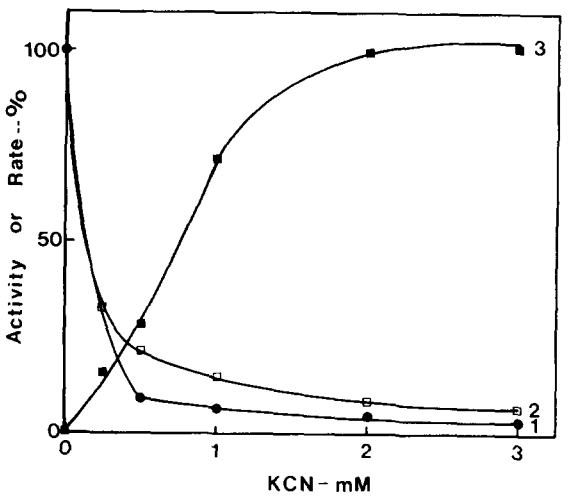


Fig. 8. Effect of KCN on formate (1) and NADH oxidase (2) activities and on the rate of reduction of the additional membrane cytochrome reduced by ascorbate-PMS in the presence of KCN (3). Oxidase activities and the rate of cytochrome reduction were determined from dual-wavleength traces as described in Materials and Methods. Oxidase activity is expressed as a percentage of the activity obtained in the absence of KCN. Rate is expressed as a percentage of the maximum rate obtained at the highest concentration of KCN.

Kinetics of reduction of the cytochromes in the presence of a mediator

Addition of PMS to the suspension of membranes before the substrate greatly increased the rate of reduction of the 'slow-phase' cytochromes (Fig. 6). The final level of reduction achieved (84% for NADH and formate, 63% for succinate) was similar to that in the absence of mediator. Presumably PMS, reduced directly by NADH or via the dehydrogenases of formate or succinate, transfers electrons directly to the 'slow-phase' cytochromes.

Addition of KCN to the cuvette in which the membrane cytochromes had been reduced to their anaerobic state level by ascorbate-PMS resulted in rapid reduction of further cytochrome (Fig. 7).

Fig. 7. Effect of KCN on the kinetics of reduction of the membrane cytochromes. Dual-wavelength measurements were carried out as described in Materials and Methods. The traces run from right to left. The vertical bar indicates absorbance units. Substrates and inhibitors were added where indicated. S, 10 µl 1 M sodium succinate; A, 25 µl 0.4 M potassium ascorbate; P, 15 µl 10 mM PMS; DQH_2, 40 µl 60 mM duroquinol; KCN, 20 µl 0.2 M KCN; D, dithionite.

This cytochrome absorbed at 556.5 nm. There was no absorption at 560–565 nm. Addition of KCN in the anaerobic state or at the end of the 'fast-phase' of reduction with succinate or duroquinol (or with NADH and formate, not shown) did not increase the rate or extent of reduction (Fig. 7). This suggests that at least part of the 'slow-phase' cytochrome made accessible to reduction by ascorbate in the presence of PMS was readily oxidized via a cyanide-sensitive oxidase by oxygen diffusing into the cuvette. Presumably, the rate of transfer of electrons from ascorbate to this cytochrome was inadequate to balance the rate of reoxidation unless the oxidase was inhibited by KCN. The relative sensitivities of NADH and formate oxidases to inhibition by KCN were compared with the effect of cyanide concentration on the rate of reduction of the extra cytochrome by ascorbate (with PMS) (Fig. 8). A significant increase in the rate at which extra cytochrome was reduced occurred only after the respiratory chain oxidase (cytochrome *o*) had been largely inhibited.

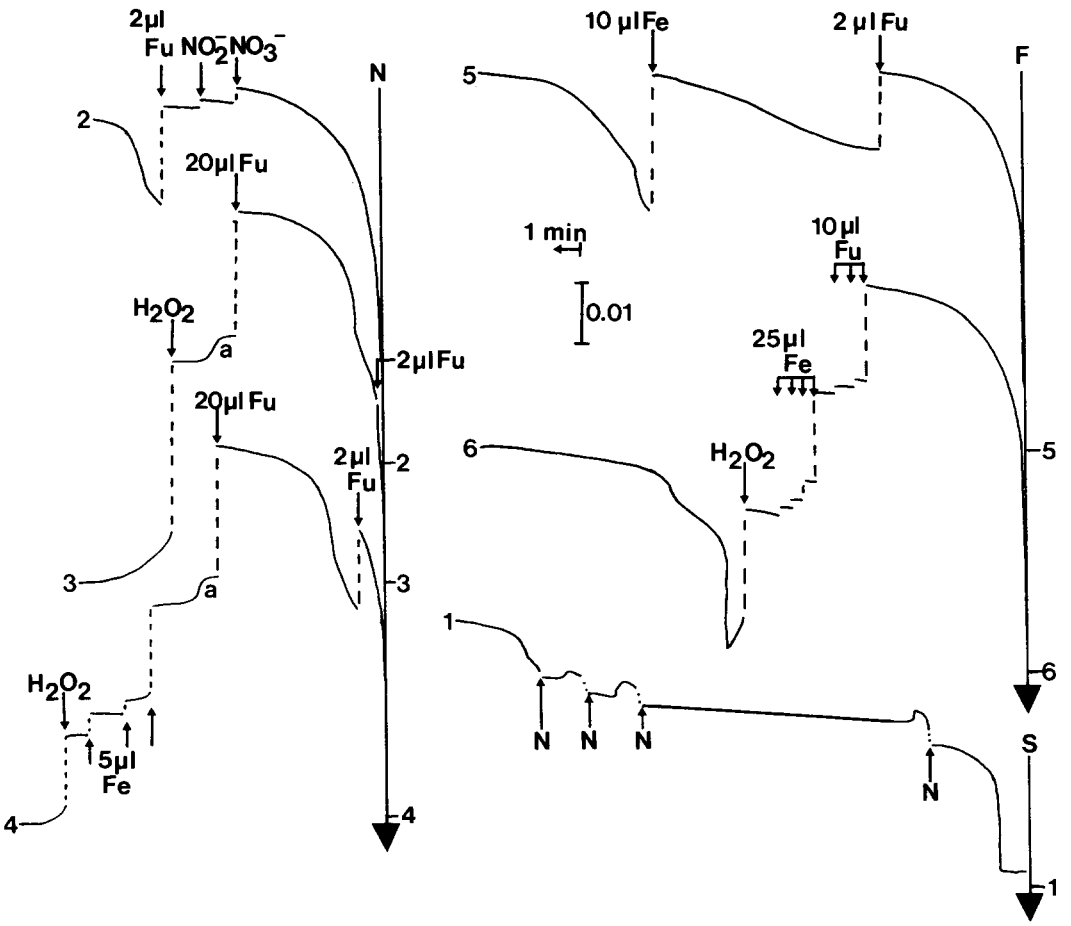


Fig. 9. Kinetics of oxidation and reduction of membrane cytochromes in the presence of fumarate and ferricyanide. Dual-wavelength measurements were carried out as described in Materials and Methods except that H_2O_2 was omitted from the buffer. The traces run from right to left. The vertical bar indicates absorbance units. Substrates were added at the arrows. F, 10 µl 1 M sodium formate; S, 10 µl 1 M sodium succinate; N, 50 µl 80 mM NADH except in trace 1 where 10 µl 80 mM NADH was added; H_2O_2, 3 µl 3% H_2O_2; NO_2^-, 5 µl 1 M $NaNO_2$; NO_3^-, 5 µl 1 M KNO_3. The volumes of M sodium fumarate (Fu) and 0.1 M potassium ferricyanide (Fe) added are shown on the traces. a, steady state level of reduction of cytochromes in the presence of excess fumarate followed by further oxidation of cytochromes when the substrate NADH was exhausted.

Characterization of 'slow-phase' cytochromes

Addition of small amounts of NADH (or formate) to a cuvette in which the cytochromes had been reduced by succinate to an anaerobic state either did not result in further reduction of the cytochrome to a higher anaerobic state level or the cytochrome was reduced only after a lag period (Fig. 9, trace 1). One possible explanation of this phenomenon was that oxidation of succinate had led to the formation of fumarate which had to be reduced by NADH or formate before further reduction of the cytochrome could occur. That both NADH and formate could reduce added fumarate is shown in Fig. 9, traces 2 and 5. Addition of fumarate, but not of NO_3^- or NO_2^-, oxidized a portion of the cytochromes which was then re-reduced when the fumarate had been reduced. The rate of reduction of fumarate by NADH (0.10 μmol/min per mg protein) was 5-fold greater than with formate (0.018 μmol/min per mg protein) although the oxidase activities were similar (NADH oxidase, 0.45 μg atom O reduced/min per mg protein; formate oxidase, 0.54 μg atom O reduced/min per mg protein). Fumarate reductase activity measured with benzyl viologen was 0.72 μmol reduced/min per mg protein.

Approx. 30–40% of the cytochrome was oxidized by excess fumarate with NADH and formate as substrates. Another 30–35% was readily oxidized if $K_3Fe(CN)_6$ was now added (Fig. 9, traces 4 and 6). If excess $K_3Fe(CN)_6$ was added without fumarate, then about 65% of the cytochrome was oxidized. H_2O_2, which generated oxygen in the presence of endogenous catalase, oxidized the remainder of the cytochrome to its aerobic state level. The cytochrome oxidized by fumarate is that which is reduced in the 'slow phase'. Thus, addition of fumarate at the end of the 'fast phase', with either NADH or formate, oxidized little cytochrome (Fig. 9, trace 3). Cytochrome was oxidized if fumarate was added midway through the 'slow phase' (Fig. 9, trace 4).

An attempt was made to identify those cytochromes oxidized by fumarate and by ferricyanide in the experiments described above. A difference spectrum between the cytochromes reduced in the presence of formate (or NADH) and those reduced in the presence of formate (or NADH) following the addition of excess fumarate showed

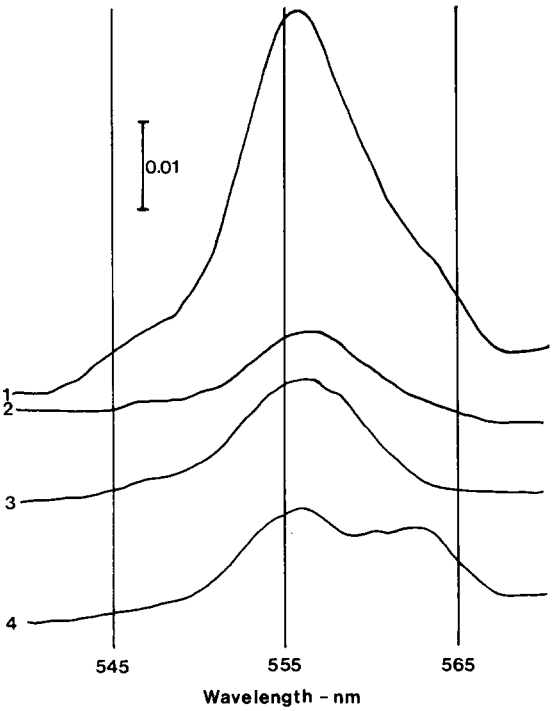


Fig. 10. Difference spectra at 77 K of membrane cytochromes oxidized by fumarate and by ferricyanide, and of the cytochromes remaining reduced in the presence of both oxidants. The vertical bar indicates absorbance units. 1, formate-reduced (for 15 min) minus H_2O_2-oxidized; 2, formate-reduced minus formate-reduced to which 20 μl M sodium fumarate was added; 3, formate-reduced to which sodium fumarate was added minus an identical sample to which 20 μl 0.1 M potassium ferricyanide was also added; 4, formate-reduced to which both sodium fumarate and potassium ferricyanide were added minus H_2O_2-oxidized.

that cytochromes contributing to the broad peak at 556.5 nm were oxidized by fumarate (Fig. 10). Ferricyanide added after fumarate oxidized cytochromes with a spectrum similar to those oxidized by fumarate. The cytochromes remaining reduced in the presence of both fumarate and ferricyanide gave two almost equal-sized peaks at 556.5 and 562.5 nm which strongly resembled the spectra of the cytochromes reduced in the 'fast phase' by ascorbate-PMS, succinate and NADH (Fig. 2).

Effect of HOQNO on cytochrome reduction

HOQNO inhibits substrate oxidation in membranes of *E. coli*. Its site of action is still not defined [1–3,8]. The effect of HOQNO on the oxidation of NADH, formate and succinate and

on the aerobic and anaerobic steady-state level of reduction of the cytochromes is shown in Fig. 11. Inhibition of oxidase activity was not accompanied by any marked decrease in the anaerobic state level of reduction of the cytochromes. The rate of reduction of cytochrome by these substrates was diminished in the presence of inhibitor. This was most obvious for the 'slow-phase' cytochromes (Fig. 1). By contrast, although HOQNO inhibited oxidation of ascorbate-PMS as shown by the increased time required to deplete dissolved oxygen in the membrane suspension (Fig. 1), this was accompanied by an increase in the rate of reduction of the 'fast-phase' cytochromes. The rate of reduction of the 'slow-phase' cytochromes was diminished.

The response of the aerobic steady-state level of reduction of the cytochromes in the presence of HOQNO depended on the substrate (Fig. 11). With NADH the aerobic steady-state level of reduction was decreased by increasing concentrations of the inhibitor. This is consistent with the inhibitory effect of HOQNO on the rate of reduction of the cytochromes. By contrast, the aerobic steady-state level of reduction with succinate was not much affected by HOQNO. Increasing concentrations of HOQNO increased the aerobic steady-state level of reduction of cytochrome by formate.

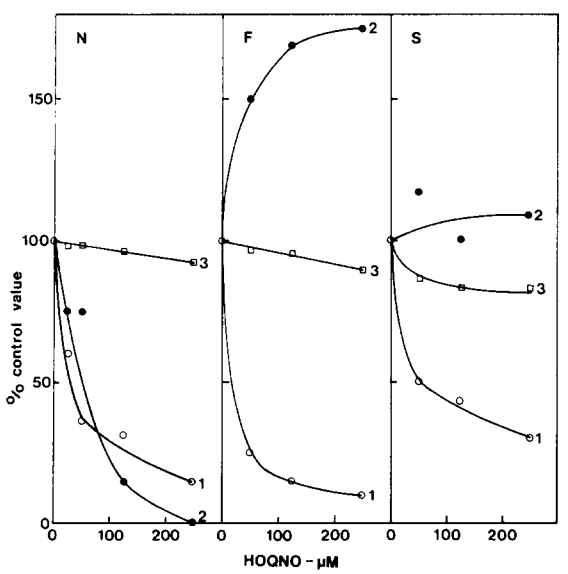


Fig. 11. Effect of HOQNO on the oxidase activity (1), aerobic steady-state (2) and anaerobic state (3) levels of reduction of the membrane cytochromes. Values were calculated from the traces of dual-wavelength kinetic experiments following addition of the substrates NADH (N), sodium formate (F) and sodium succinate (S). Oxidase activity was calculated from the time required to deplete dissolved oxygen in the cuvette as shown by the initiation of cytochrome reduction on anaerobiosis.

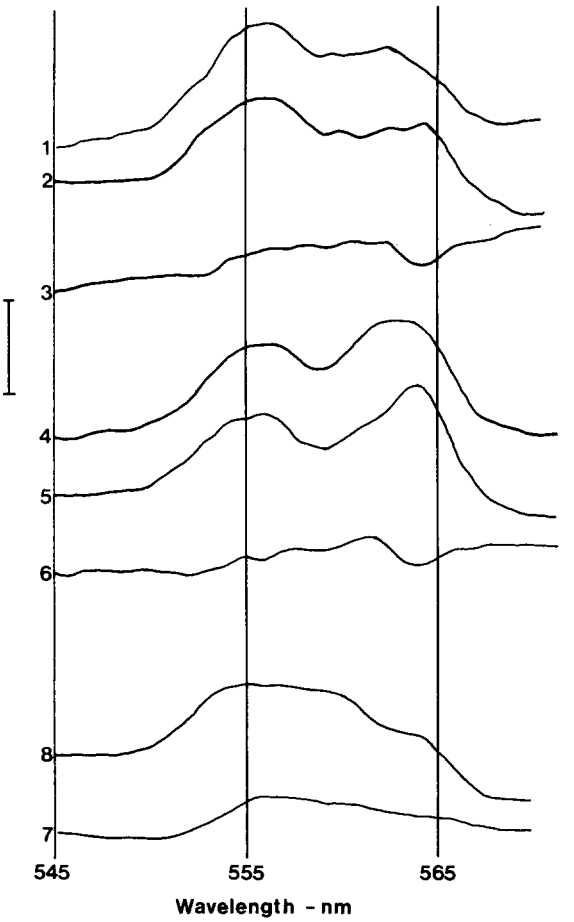


Fig. 12. Difference spectra at 77 K of membrane cytochromes reduced in the presence and absence of 0.1 mM HOQNO. The phase of reduction being measured can be determined by reference to the kinetic traces in Fig. 1. The vertical bar represents an absorbance of 0.01 units for traces 1–6 and 0.005 units for traces 7 and 8. 1, succinate-reduced (for 1 min) minus H_2O_2-oxidized; 2, succinate with HOQNO-reduced (for 2 min) minus H_2O_2-oxidized; 3, calculated difference between spectra 1 and 2; 4, ascorbate-PMS (P-A)-reduced (for 0.75 min) minus H_2O_2-oxidized; 5, P-A reduced (for 2 min) minus H_2O_2 oxidized; 6, calculated difference between spectra 4 and 5; 7, formate-reduced (aerobic steady state) minus H_2O_2-oxidized; 8, formate with HOQNO-reduced (aerobic steady state) minus H_2O_2-oxidized.

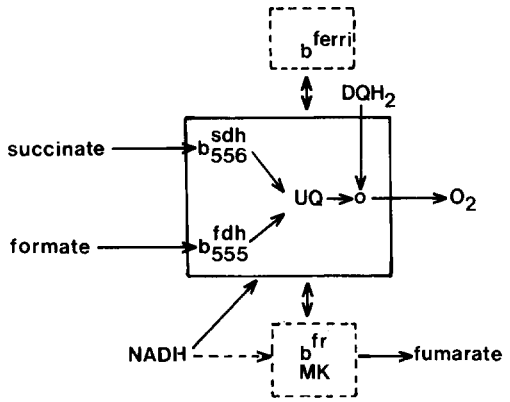


Fig. 13. Cytochrome pools of *E. coli* membranes. Electrons are transferred from the substrates succinate and formate into the ubiquinone (UQ) pool via the cytochromes associated with their dehydrogenases (b_{556}^{sdh}, b_{555}^{fdh}). Ubiquinol is oxidized by cytochrome *o*, which can also be reduced by duroquinol (DQH_2). These cytochromes constitute the 'fast-phase' cytochromes. The 'slow-phase' cytochromes b^{ferri} (oxidized by ferricyanide, but not by fumarate) and b^{fr} (oxidized by fumarate) are reduced through their connection with the 'fast-phase' cytochromes, although NADH can also reduce b^{fr} directly, probably via menaquinone (MK). See text for further discussion.

The effect of HOQNO on the reduction of specific *b* cytochromes was examined by difference spectroscopy (Fig. 12). Spectra measured at the end of the 'fast phase' showed that HOQNO increased the extent of reduction at about 564 nm by ascorbate-PMS and decreased that between 555-560 nm. This was particularly evident in the difference spectrum between samples recorded with and without the addition of the inhibitor. A trough at 564 nm was evident in these spectra. Similar results were obtained with succinate. Measurement of the spectrum of the cytochromes reduced in the aerobic steady state was difficult because of the rapidity with which formate oxidation depleted the dissolved oxygen in the membrane suspension. However, the spectrum shown in Fig. 12, trace 7 is typical of those obtained. It shows that cytochromes absorbing at 556 nm were more reduced than those at 564 nm. The presence of HOQNO increased the aerobic steady-state level of reduction of the cytochromes absorbing at 564 nm as well as for those obsorbing between 554 and 560 nm. Thus, the effect was similar to that seen with ascorbate-PMS and with succinate, but with the addition of an increased reduction of those cytochromes absorbing at about 556 nm.

Discussion

The *cydA* mutant GR19N lacks the components of the cytochrome *d* complex, that is, cytochromes *b*-558, *b*-595 and *d* [21]. In spite of this, the results presented in this paper indicate that membranes of cells of this strain grown aerobically on a proline-amino acid medium have a complex content of cytochromes. Kinetic studies using dual-wavelength spectrophotometry showed that the cytochromes could be grouped into two groups, those rapidly reduced and those slowly reduced by the substrates NADH, formate, succinate and ascorbate-PMS (Fig. 13). The cytochromes reduced in the 'slow phase' gave a broad peak at about 556.6 nm, whereas those reduced in the 'fast phase' showed two broad peaks centered at 556.5 and 562.5 nm in substrate-reduced minus oxidized difference spectra. The latter peaks were composed of several incompletely separated components having absorption maxima (at 77 K) at 554–555, 556–557.5, 560–561.5 and 563.5–564.5 nm. In addition, the small shoulder on the short-wavelength side of the major absorption band appeared to be composed of two overlapping peaks at 548.5 and 551.5 nm. These were not examined further. There was some variation in the positions of the absorption maxima which may be due to overlapping of the absorption peaks.

Three of the peaks reduced in the 'fast phase' can be attributed to cytochrome *o*. Purified cytochrome *o* in dithionite- or duroquinol-reduced minus oxidized difference spectra gave an alpha-band composed of fused peaks with maxima at 555, 557 and 563.5 nm (Fig. 3) which could be resolved into peaks at 554.5, 557.5 and 563.5 nm in fourth-order finite difference spectra. Kita et al. [5], Hackett and Bragg [22] and Kranz and Gennis [25] have presented spectra of purified cytochrome *o* measured at 77 K which showed absorption peaks at 555 and 562 nm. The presence of three peaks in the present preparation was probably due to the improved resolution obtained by the use of a narrow band width and by averaging of spectra.

The small peak at 560–561.5 nm seen with membranes may be the cytochrome *b*-561 (midpoint oxidation-reduction potential (E_m), +20 mV) purified by Murakami et al. [13]. This cytochrome is present in low amounts in the membranes from normal strains, and would be expected to be reduced by duroquinol (E_m, 0 mV),

as was observed in our experiments.

Two other cytochromes probably contributed to the difference spectra of cytochromes reduced in the 'fast phase' but their presence would not be obvious because of overlapping with the 554.5 and 557.5 nm peaks of cytochrome *o*. The appreciable succinate oxidase activity of the membranes would be consistent with the presence of cytochrome *b*-556, a cytochrome recently found to be coded by the *sdh* operon and having a midpoint oxidation-reduction potential of −45 mV in purified detergent-solubilized preparations [12,11].

The high formate oxidase activity of the membranes suggested that the cytochrome associated with formate dehydrogenase was also present. This cytochrome has an absorption maximum at 555.5 nm (77 K) and an oxidation-reduction potential of about −110 mV [14–16]. Consistent with the presence of this cytochrome was the higher aerobic steady-state level of reduction, observed at 556 nm, by formate than by NADH, in spite of their similar oxidase activities.

The cytochromes reduced in the 'slow phase' have maxima which overlap the broad peak at about 556.5 nm of cytochrome *o* and thus could not be separated from it spectroscopically. The slowness of reduction of the cytochromes in this group was due to the slowness at which electrons were delivered to the cytochromes, probably from the quinone pool (Fig. 13). The rate or extent of reduction of these cytochromes by NADH and formate was not increased if the reaction was carried out under oxygen-free nitrogen or in the presence of KCN to block the terminal oxidase. However, with ascorbate, which was not able to provide electrons to PMS at a rate sufficient to reduce the 'slow-phase' cytochromes fully in the presence of oxygen diffusing into the sample, inhibition of cytochrome *o* with cyanide [26] resulted in the rapid reduction of the previously reduced 'slow phase' cytochromes. Electrons from the 'slow-phase' cytochromes must therefore be transferable to cytochrome *o* as proposed in Fig. 13.

Two pools of cytochromes were detected within the 'slow-phase' cytochromes. Excess fumarate oxidized about one-third of the total cytochromes. Addition of excess ferricyanide after fumarate oxidized another one-third. Approximately one-third of the total cytochrome constituted the 'fast-phase' cytochrome pool. These results suggested that about one-half of the 'slow-phase' cytochromes was closely associated with the fumarate reductase system. The fumarate reductase activity of the membranes was high. The involvement of *b* cytochrome in the fumarate reductase pathway is controversial. Although the experiments of Singh and Bragg showed that electron transfer between substrate and fumarate could occur in the absence of cytochrome, substrate-fumarate reductase activities were much higher if cytochrome was present [27]. Kröger has demonstrated that there is a specific *b*-cytochrome in the fumarate reductase system of *Wolinella succinogenes* [28]. However, Weiner and his coworkers [29], who have extensively studied the purified fumarate reductase of *E. coli*, found that the reductase could be reduced directly in the absence of cytochrome by the quinone analog 2,3-dimethyl-1,4-naphthoquinone.

Fumarate was reduced less readily by formate than by NADH, although NADH and formate oxidase activities were similar. This may be explained if formate dehydrogenase reduces ubiquinone only whereas NADH dehydrogenase reduces both ubiquinone and menaquinone (Fig. 13). Menaquinone has been shown to be associated with the fumarate reductase pathway in *E. coli* [27].

No function has been associated with that cytochrome which was oxidized by ferricyanide following prior oxidation of part of the 'slow phase' cytochromes by fumarate. Ferricyanide could also oxidize the fumarate-oxidizable cytochrome. Its apparent inability to oxidize the 'fast-phase' cytochromes was due to the rapid rate at which these were reduced by substrate. Thus, the limit of oxidation by ferricyanide was the steady-state level maintained by the relative rates of reduction and oxidation.

HOQNO inhibited substrate oxidation through the respiratory chain. Several effects on cytochrome reduction were observed. Formate normally maintained a higher aerobic steady-state level of reduction of the cytochromes than NADH. The extent of reduction of cytochromes absorbing at about 556 nm was increased by HOQNO in the formate maintained aerobic steady-state. It is likely that this cytochrome was the low-potential cytochrome specifically associated with formate dehydrogenase [16]. Enoch and Lester [15] and Sánchez Crispín et al. [30] have shown previously

that HOQNO blocked electron transfer from this cytochrome to the remaining cytochrome in the formate-nitrate reductase pathway of *E. coli*. HOQNO decreased the rate of reduction by formate, NADH and succinate of the *b*-type cytochromes in the 'fast phase'. However, its most noticeable effect was to decrease the rate of reduction of the cytochromes of the 'slow phase'. These results suggest that HOQNO inhibited electron transfer from the ubiquinone pool to the cytochromes. This is consistent with the decreased aerobic steady state level of reduction of the cytochromes by NADH in the presence of the inhibitor (Fig. 11). It was supported also by the inhibitory effect of HOQNO on the reduction of the cytochromes by duroquinol, a ubiquinol analog.

HOQNO increased the level of reduction of the cytochrome absorbing at 564 nm at the end of the 'fast phase' of reduction. This is consistent with a second site of action of HOQNO between this cytochrome and oxygen. By contrast with the other substrates, HOQNO increased the rate of cytochrome reduction with ascorbate-PMS. An increased extent of reduction of cytochrome *b*-564 was also observed with this substrate. Thus, PMS must introduce electrons into the respiratory chain prior to cytochrome *b*-564 but after the inhibition site affecting electron transfer from the quinone pool to the cytochromes. Cytochrome *b*-564 is a component of the cytochrome *o* complex. The pathway of electrons and the oxygen-reacting species within this complex are unknown (see Introduction).

The results described above suggest that there are two sites in the respiratory chain at which HOQNO can interact – one at the level of the ubiquinone pool and a second site in the cytochrome *o* complex. Alternatively, a single site of action of HOQNO is possible if a Q-cycle mechanism or *b* cycle mechanism for the organization of the *b* cytochromes and ubiquinone is present [1,2,8].

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