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Sequence of *b* Cytochromes Relative to Ubiquinone in the Electron Transport Chain of *Escherichia coli*

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A ubiquinone-deficient mutant, carrying mutations in two genes affecting ubiquinone biosynthesis, has been used, in comparison with a normal strain, to determine the sequence of some of the components of the electron transport chain of *Escherichia coli.* The amounts of cytochromes reduced during aerobic steady-state conditions were estimated by comparing low-temperature difference spectra of normal or ubiquinone-deficient membranes with either D-lactate or reduced nicotinamide adenine dinucleotide as substrate. From the amounts of cytochromes reduced it was concluded that ubiquinone functions at two sites, one site being between the dehydrogenases and cytochromes and the second site being after cytochromes b_{562} and b_{556} but before cytochromes b_{558}, *d*, and *o*. The scheme proposed is discussed in relation to the Mitchell protonmotive ubiquinone cycle.

Aerobically grown *Escherichia coli* forms a number of spectrophotometrically distinguishable cytochromes. Shipp (20) identified three *b*-type cytochromes, which had absorption maxima at 556, 558, and 562 nm when measured at 77°K. In addition, Castor and Change (3) demonstrated the presence of cytochrome *o*, a *b*-type cytochrome, by its characteristic photochemical action spectrum when complexed with carbon monoxide. Cytochrome *d*, which has a peak of absorption at 627 nm when measured at 77°K, also functions as an oxidase (3), and this cytochrome, together with cytochrome b_{558}, appears to be regulated coordinately, both cytochromes being formed in the late log phase or early stationary phase of growth (11, 18).

Although three *b* cytochromes have been identified spectroscopically, their sites of interaction in the electron transport pathway relative to other components have not been characterized. Hendler et al. (12) determined that the midpoint potentials of the *b* cytochromes were −50, +110, and +220 mV. Pudek and Bragg (19), however, detected two major components with midpoint potentials of +36 and +165 mV and attributed the higher-potential cytochrome to cytochrome b_{558} and the lower-potential cytochrome to cytochrome b_{556}.

The relative sequence of cytochrome b_1 and ubiquinone in the electron transport chain of *E. coli* has been studied by using a ubiquinone-deficient mutant, and the conclusion has been drawn that ubiquinone functions both before and after cytochrome b_1 (7).

More recently, this proposal of two sites of ubiquinone function in the *E. coli* respiratory chain has attracted added interest in view of the proposal by Mitchell (14–16) of a protonmotive ubiquinone cycle. In the present paper, we propose an electron transport scheme in which the order of the various *b* cytochromes relative to ubiquinone has been based on results obtained using a quinone-deficient mutant carrying mutations in two genes affecting ubiquinone biosynthesis.

MATERIALS AND METHODS

Chemicals. Chemicals were of the highest purity available commercially and were not further purified. Duroquinol was prepared by reduction of an ethanolic solution of duroquinone with sodium borohydride. After acidification with dilute hydrochloric acid, the duroquinol was crystallized from water-ethanol, filtered, washed with water, and dried.

Organisms. The organisms used are derivatives of *E. coli* K-12: strain AN704 *ilvC argH hemA entA leu* and its derivative strain AN750 *ubiA ubiB hemA entA leu.* The genetic nomenclature used is that described by Bachmann et al. (2).

Transduction technique. The technique for transduction experiments, in which the generalized transducing bacteriophage P1*kc* was used, was that described by Pittard (17).

Media and growth of organisms. The minimal medium used was that described by Gibson et al. (9). Growth supplements were added where required, as sterile solutions, at the following final concentrations; glucose, 30 mM; thiamine hydrochloride, 0.2 μM; 2,3-dihydroxybenzoate, 40 μM; L-arginine-hydrochloride, 0.8 mM; L-isoleucine, 0.3 mM; L-valine, 0.3 mM; L-

leucine, 0.6 mM; 5-aminolevulinic acid, 20 μM.

Cells for the preparation of membranes were grown in 14-liter New Brunswick fermenters as described previously (7).

Preparation of cell membranes. Membranes were prepared as described previously (5). Briefly, washed cells were disintegrated by using a Sorvall-Ribi cell fractionator, and the membranes were separated by ultracentrifugation and suspended in a 0.1 M *N*-tris(hydroxymethyl)methyl-2-aminomethane-sulfonic acid buffer system adjusted to pH 7.0 with NaOH and containing magnesium acetate, sucrose, ethyleneglycol-bis(β-aminoethyl ether)-*N,N*-tetraacetic acid. Protein concentrations were determined by using the Folin phenol reagent (13) with bovine serum albumin (fraction V; Sigma Chemical Co., St. Louis, Mo.) as the standard.

Oxidase activities. Oxygen uptakes by membrane preparations were measured with an oxygen electrode as described previously (7).

Difference spectra measured at 77°K. Recordings of difference spectra measured at 77°K were made with an Aminco-Chance DW-2 spectrophotometer with a liquid N_2 low-temperature accessory (American Instrument Co., Inc., Silver Spring, Md.). Difference spectra were recorded by using 2-mm light path cuvettes, a spectral band width of 1.0 nm, a scanning speed of 0.5 nm/s, and the medium time-response setting. At high sensitivities, the slow time-response setting was used, and the scanning speed was reduced to 0.1 nm/s.

Membranes were diluted in the *N*-tris(hydroxymethyl)methyl-2-aminomethane-sulfonic acid buffer system to a protein concentration of about 10 mg/ml; additions were made as described in the Figure legends. The samples were then frozen by immersion in liquid N_2. Estimates of the relative amounts of cytochromes present, based on the absorption peak heights, were reproducible to within 10%.

RESULTS

Preparation of strains. In previous studies with ubiquinone-deficient mutants of *E. coli* K-12, it proved difficult to maintain the purity of cultures used for the preparation of membranes (7). To overcome this problem, a strain carrying two mutations affecting different steps in the biosynthesis of ubiquinone was isolated after a series of transduction experiments. The starting strain (AN704) carries mutations in the *ilvC, argH,* and *hemA* genes. Strain AN708 was prepared from strain AN704 by cotransduction of a mutation in the *metA* gene with the *argH* gene. The mutant allele *ubiA351* was cotransduced into strain AN708 with the *metA* gene to give strain AN725. Strain AN725 is unable to grow on succinate-minimal medium due to the lack of ubiquinone but is able to grow on this medium in the presence of high concentrations of 4-hydroxybenzoate, an intermediate in ubiquinone biosynthesis (6). A mutation in the *metE* gene was then cotransduced with the *ilvC* gene into strain AN725 to give strain AN729. The *ubiB409* mutant allele was then cotransduced with the *metE* gene into strain AN729 to give strain AN750. The $metE^+$ transductants obtained in this last transduction were screened for the presence of the *ubiB409* allele by the inability of such strains to grow on succinate-minimal medium supplemented with a high concentration of 4-hydroxybenzoate.

The two strains used throughout this paper are the parent strain, AN704, and the ubiquinone-deficient strain AN750, which carries the mutant alleles *ubiA351* and *ubiB409*. Strain AN750 did not revert during growth of cells for the preparation of membranes and did not form ubiquinone, although trace amounts of the ubiquinone-biosynthetic intermediate, 2-octaprenylphenol, could be detected.

Electron transport rates. The reduced nicotinamide adenine dinucleotide (NADH) and D-lactate oxidase rates were, as expected, lower in membranes from the ubiquinone-deficient strain, AN750, than in membranes from the normal strain, AN704, and were stimulated by the addition of ubiquinone (coenzyme Q_1) (Table 1). The effect of the ubiquinone deficiency was greater on the NADH oxidase system than on the D-lactate oxidase system, the value for the NADH oxidase system in AN750 membranes being only 3% of that in AN704 membranes. Duroquinol oxidase activity was higher in membranes from strain AN750 (*ubi*) than in AN704 membranes and was unaffected by the addition of ubiquinone (coenzyme Q_1), although the addition of ubiquinone did cause stimulation of the duroquinol oxidase in membranes from strain AN704 (Table 1). The duroquinol oxidase activity was, however, dependent on the pres-

TABLE 1. *Comparison of the oxidase activities in membranes from strains AN704 and AN750 (ubi)*

Substrate + additions[a]	Oxygen uptake (ng-atom of O per min per mg of protein) by membranes from:	
	AN704	AN750
NADH	770	23
NADH + Q_1	1,520	1,042
D-lactate	587	170
D-lactate + Q_1	962	1,290
Duroquinol	403	618
Duroquinol + Q_1	710	610

[a] Ubiquinone (coenzyme Q_1) was added in ethanol at a final concentration of 80 μM which gave maximal oxidase rates for each strain. NADH, D-lactate, and duroquinol (in ethanol) were at final concentrations of 1 mM. For details, see the text.

ence of cytochromes, as membranes prepared from either strain AN704 or strain AN750 (*ubi*) starved of 5-aminolevulinate completely lost the duroquinol oxidase activity.

Cytochrome reduction by various substrates. The reduced-minus-oxidized difference spectra with sodium dithionite as reductant obtained by using membranes prepared from either the normal strain or the ubiquinone-deficient strain indicate that the lack of ubiquinone has no significant effect on cytochrome content. The spectra shown in Fig. 1 indicate that, in these particular membrane preparations, the cytochrome *d* (absorption peaks, 439 and 625 nm; trough, 645 nm) and cytochrome b_{558} contents were higher in strain AN704 than in strain AN750 (*ubi*). This is presumably due to the fact that the AN704 cells used for this particular membrane preparation were harvested later in logarithmic phase than were those of strain AN750 (*ubi*). The reduced-minus-oxidized difference spectra with D-lactate and NADH as substrates were similar to those obtained for the reduced-minus-oxidized difference spectra with dithionite (Fig. 1), with 85 to 95% of each of the cytochromes being reduced enzymatically.

Duroquinol oxidase activity was higher in membranes from strain AN750 (*ubi*) than in membranes from strain AN704 (Table 1). It was of interest, therefore, to examine the reduced-minus-oxidized difference spectra, with duroquinol as substrate, of the membranes prepared from both strain AN750 (*ubi*) and strain AN704 (Fig. 2). These spectra may be directly compared with those shown in Fig. 1. Comparing the Soret peaks of absorption of cytochrome *d* (439 nm) and of cytochromes *b* (428 nm) it is clear that cytochrome *d* was more reduced than the *b* cytochromes. Calculating the percent reduction of the cytochromes by using the absorption in the α bands, where there is no interference from other absorbing species, cytochrome *d* (peak, 625 nm, trough, 645 nm) was about 70% reduced in strain AN704 and about 80% reduced in strain AN750 (*ubi*). The absorption in the 556- to 558-nm region of the spectrum indicates that about 30% of the *b* cytochromes in strain AN750 and about 40% of the *b* cytochromes in strain AN704 were reduced. However, since cytochrome *d* was almost completely reduced by duroquinol, it is likely that cytochrome *o* was also reduced by duroquinol. Thus, a significant proportion of the absorption in the 428- and 556- to 558-nm regions of the spectrum may have been due to reduced cytochrome *o*.

Reduction of cytochromes in the aerobic

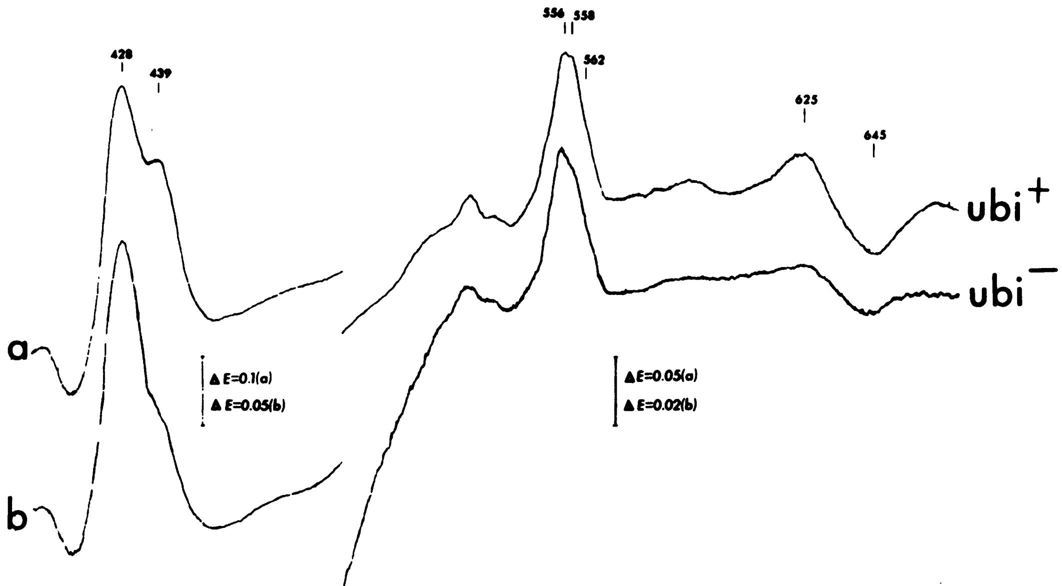

FIG. 1. *Reduced-minus-oxidized difference spectra with dithionite as reductant recorded at 77°K with membranes from normal and ubiquinone-deficient E. coli. Membranes, prepared as described in the text, were diluted to about 10 mg of protein per ml. Before the samples were frozen in liquid N_2, about 2 mg of sodium dithionite were mixed with the membranes in the sample cuvette, and H_2O_2 (about 1 mM) was mixed with the membranes in the reference cuvette and then incubated for 20 s at room temperature. The spectra were scanned from 400 to 670 nm, and the wavelengths (in nanometers) of the absorption peaks are as shown. (a) Membranes from strain AN704, 14.3 mg of protein per ml; (b) membranes from strain AN750 (ubi), 8.1 mg of protein per ml.*

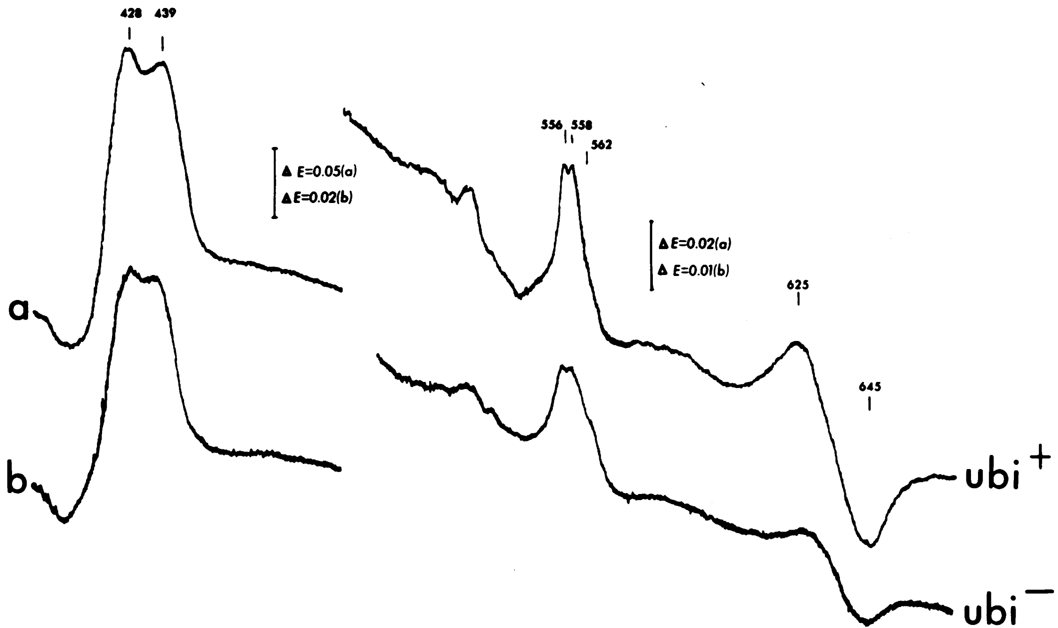

FIG. 2. *Reduced-minus-oxidized difference spectra with duroquinol as substrate recorded at 77°K with membranes from normal and ubiquinone-deficient E. coli. The conditions were as described in the legend to Fig. 1, except that, instead of dithionite, 10 μl of an ethanolic solution of duroquinol (0.1 M) was added to the sample cuvette, and 10 μl of ethanol was added to the reference cuvette, which contained H_2O_2 (about 1 mM), and then incubated at room temperature for 60 s before freezing in liquid N_2. The spectra were scanned from 400 to 670 nm, and the wavelengths (in nanometers) of the absorption peaks are as shown. (a) Membranes from strain AN704, 14.3 mg of protein per ml; (b) membranes from strain AN750 (ubi), 8.1 mg of protein per ml.*

steady state. During steady-state electron flow, with both substrate and oxygen present in excess, the cytochromes become partially reduced, and the amount of this reduction is dependent on the relative dehydrogenase and oxidase activities. Thus, it would be anticipated that cytochrome *d* would be almost fully oxidized during steady-state conditions, whereas cytochromes closer to the dehydrogenases would be partially reduced. Moreover, if electron flow is inhibited, then any components between the dehydrogenase and the point of inhibition would become more reduced, and components on the oxygen side of the block would become more oxidized (4). Using the ubiquinone deficiency as a block in electron flow, we have directly estimated, by low-temperature spectrophotometry, the relative amounts of cytochromes reduced during steady-state conditions.

In membranes from strains AN704 and AN750 (*ubi*), there was no detectable reduction of cytochrome *d* (peaks, 439 and 625 nm; trough, 645 nm) in the aerobic steady state with either NADH or D-lactate as substrate, as would be expected under aerobic steady-state conditions (Fig. 3 and 4). Both substrates reduced about 5 to 10% of the cytochromes *b* (peaks, 428 and 556 to 562 nm) in membranes from strain AN704 in the aerobic steady state, although the particular *b* cytochrome reduced depended on the substrate added: cytochromes b_{556} and b_{562}, but not b_{558}, were reduced by NADH (Fig. 3), whereas D-lactate predominantly reduced cytochrome b_{556} (Fig. 4).

The situation was very different in membranes from the ubiquinone-deficient mutant, AN750. With NADH as substrate, 50 to 60% of the cytochrome b_{556} was reduced under aerobic steady-state conditions. The amount of cytochrome b_{562} reduced under these conditions is difficult to estimate, but it was probably also about 50 to 60% reduced. The reduction of cytochrome b_{558} under aerobic steady-state conditions could not be detected, although the large amounts of cytochromes b_{556} and b_{562} reduced under these conditions would make it difficult to detect a low level of b_{558} reduction. The addition of 80 μM ubiquinone (coenzyme Q_1) to the AN750 membranes changed the amount of cytochrome b_{556} reduced by NADH in the aerobic steady state to an amount similar to that obtained with membranes from strain AN704. However, the lack of reduction of cytochrome b_{562} in the AN750 membranes with added coenzyme Q_1 as compared with the AN704 membranes indicates that added coenzyme Q_1 is not

exactly analogous to the endogenous coenzyme Q_8 present in strain AN704.

DISCUSSION

A scheme for the function of ubiquinone in the electron transport chain of *E. coli* has been previously proposed (7), based on results obtained using a ubiquinone-deficient mutant. This scheme postulated two sites of ubiquinone interaction with the electron transport chain, one before and one after the *b* cytochromes. A subsequent scheme, suggested by Poole and Haddock (18), placed ubiquinone as a single pool before the *b* cytochromes. In general, the results reported in this paper support the scheme of Cox et al. (7) and permit a more detailed scheme to be proposed, as summarized in Fig. 5. Flavins and iron-sulfur proteins have not been investigated in the present work and are, therefore, not included in the sequence depicted in Fig. 5.

It is clear from the steady-state data presented in Fig. 3 that ubiquinone functions after cytochromes b_{556} and b_{562}: in the steady-state spectrum with NADH as substrate, these two cytochromes are at least 50 to 60% reduced in the membranes prepared from the ubiquinone-deficient strain as compared with about a 5 to 10% reduction in membranes prepared from the normal strain. On the addition of ubiquinone (coenzyme Q_1) to the membranes prepared from the ubiquinone-deficient strain, the percent reductions of cytochromes b_{556} and b_{562} returned to the normal levels.

A comparison of the steady-state reduction

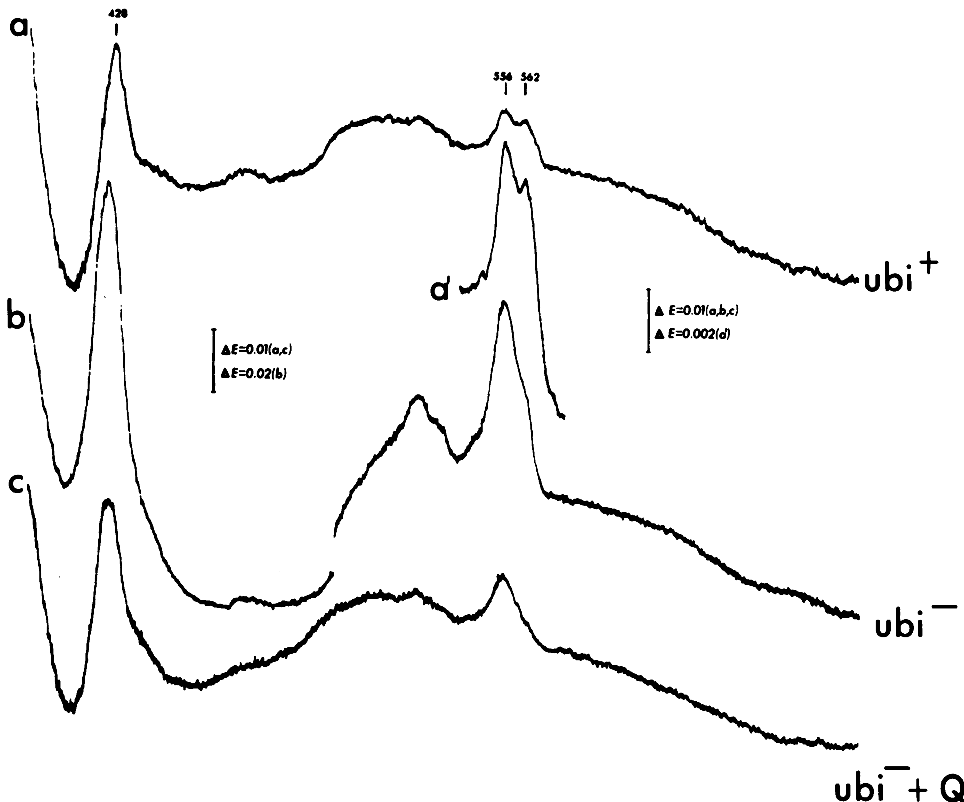

FIG. 3. *Steady-state-minus-oxidized difference spectra with NADH as substrate recorded at 77°K with normal and ubiquinone-deficient membranes of E. coli. H_2O_2 (about 5 mM) was added to 2.5 ml of membranes at room temperature, and then 1.0-ml portions of this membrane suspension were transferred to the cuvettes. The sample cuvette contained 40 μl of 0.1 M NADH, and the reference cuvette contained 40 μl of water. After mixing, the membranes were incubated at room temperature for 30 s (traces a, a', and c) or 180 s (trace b) and then frozen in liquid N_2. In trace c, 10 μl of an ethanolic solution of ubiquinone (coenzyme Q_1) (40 mM) was added before the addition of H_2O_2. The spectra were scanned from 400 to 670 nm, and the wavelengths (in nanometers) of the absorption peaks are as shown. (a) Membranes prepared from strain AN704, 14.3 mg of protein per ml; (a') as for (a), but recorded at higher sensitivity with the high-sensitivity recording conditions described in the text; (b) membranes prepared from strain AN750 (ubi), 8.1 mg of protein per ml; (c) as for (b), except that ubiquinone (coenzyme Q_1) was added.*

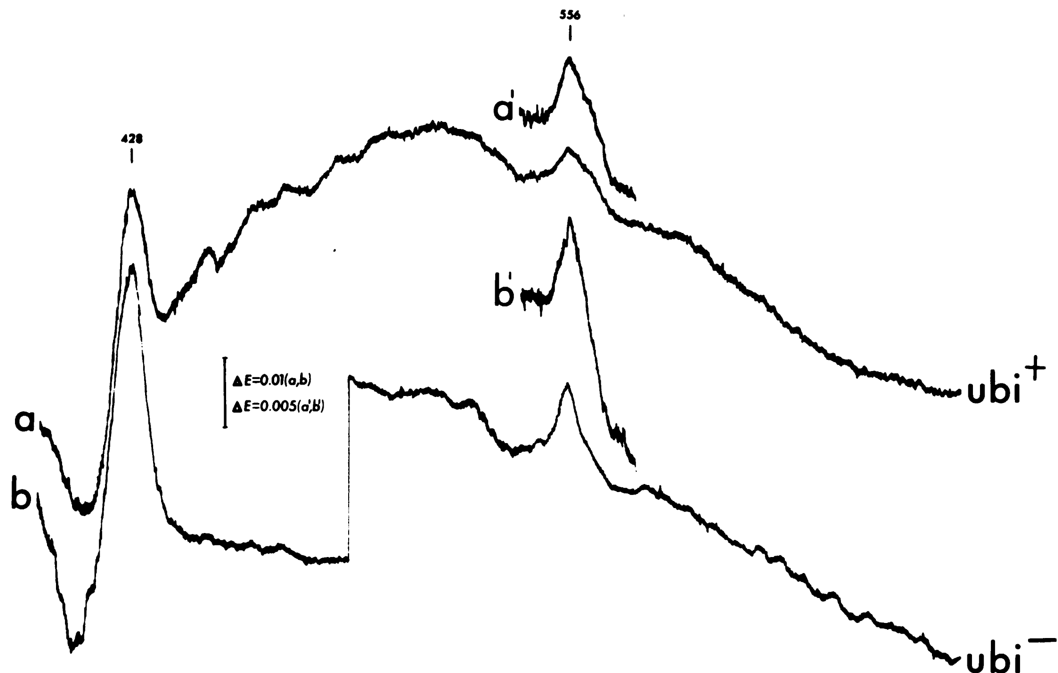

FIG. 4. *Steady-state-minus-oxidized difference spectra with D-lactate as substrate recorded at 77°K with normal and ubiquinone-deficient membranes from E. coli. The conditions were similar to those described in the legend to Fig. 3, except that, instead of NADH, a 10-μl addition of 0.5 M D-lactate was added to the sample cuvette, and 10 μl of water was added to the reference cuvette. The spectra were scanned from 400 to 670 nm, and the wavelengths (in nanometers) of the absorption peaks are as shown. (a) Membranes from strain AN704, 14.3 mg of protein per ml; (a') as for (a), but recorded at higher sensitivity, using the high-sensitivity recording conditions described in the text; (b) membranes from strain AN750 (ubi), 8.1 mg of protein per ml; (b') as for (b), but recorded at higher-sensitivity recording conditions described in the text.*

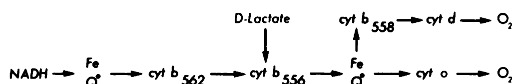

FIG. 5. *Sequence of the b cytochromes relative to ubiquinone in the electron transport chain of E. coli. Abbreviations: cyt, cytochrome; Q˙, ubisemiquinone.*

levels of cytochrome b_{562} with NADH or D-lactate as substrate in membranes from both strains reveals that this cytochrome is more strongly reduced by NADH than by D-lactate. This is more obvious with membranes prepared from AN704, in which more cytochrome b_{562} is present, but a similar result has been obtained with membranes prepared from a different batch of the ubiquinone-deficient strain that contained a relatively greater amount of cytochrome b_{562}. Thus, it is likely that cytochrome b_{562} precedes cytochrome b_{556} and that cytochrome b_{556} is the point of entry of electrons into the respiratory chain from the D-lactate dehydrogenase.

It is obviously very difficult to determine accurately the percentage of cytochrome b_{562} reduced in the NADH steady-state spectra in membranes from the ubiquinone-deficient strain, but it appears to be of the same order as that of cytochrome b_{556}. Presumably, the reduction of these two cytochromes could be due to a leak through the first site of quinone interaction in the respiratory chain. Although not shown, we observed that the steady-state reduction of cytochromes b_{556} and b_{562} took a longer time to be achieved in membranes prepared from the ubiquinone-deficient strain than in normal membranes. At intermediate stages in the NADH steady-state reduction, the relative reductions of cytochromes b_{556} and b_{562} remained the same. In view of the slow reduction of these cytochromes and the fact that only about 60%, and not 100%, of the cytochrome b_{556} was reduced, we conclude that ubiquinone functions both before and after this cytochrome. However, due to the difficulties involved in the estimation of the amount of cytochrome b_{562} reduced, the possibility cannot be ruled out that cytochrome b_{562} precedes the first ubiquinone site.

It is of considerable significance that no reduction of cytochrome *d* and, in particular, cytochrome b_{558} was detected in any of the steady-

state spectra, even with membranes prepared from the ubiquinone-deficient mutant. This indicates that cytochromes b_{558} and *d* function after the ubiquinone. Cytochromes b_{558} and *d* appear to be synthesized coordinately and appear together during the late logarithmic phase of growth (11), during growth in the presence of cyanide (1), during anaerobic growth either in the presence or in the absence of nitrate (10), or during aerobic sulfate-limited chemostat culture (18). Thus, it appears that cytochrome b_{558} is associated with cytochrome *d* rather than with cytochrome *o*.

The data obtained with duroquinol as the reducing agent indicate that this substrate feeds in primarily at the level of cytochrome *d* and, presumably, cytochrome *o*. The high duroquinol oxidase rate in the ubiquinone-deficient mutant that is unaffected by the addition of coenzyme Q_1 would, similarly, suggest that duroquinol feeds electrons into the electron transport sequence after the second ubiquinone site. According to the scheme proposed, the duroquinol reduced-minus-oxidized difference spectrum obtained with membranes from the ubiquinone-deficient strain should be a mixture of cytochromes *d* and *o*. The absorption in the 556- to 560-nm region presumably includes a contribution from reduced cytochrome *o*. It is of interest that cytochrome b_{558}, although present at a lower level in the ubiquinone-deficient strain compared with the normal strain, is significantly reduced by duroquinol. Some absorption in the *b* region may be due to reduction of cytochromes b_{556} and b_{562}, particularly in membranes from the normal strain, where a leak back along the chain would not be prevented by the lack of ubiquinone. Endogenous substrates would, however, also contribute to the reduction of the *b* cytochromes after anaerobiosis had occurred.

The conclusions drawn from the steady-state data discussed above and summarized in Fig. 5 are in general agreement with the oxidation-reduction midpoint potentials of the cytochromes reported by Pudek and Bragg (19) and Hendler et al. (12). Pudek and Bragg (19) showed that when the spectrum of cytochrome *b* (at room temperature) was analyzed at +140 mV, the high-potential component (with a midpoint potential of about +165 mV) had an absorption of 560 nm, whereas when the spectrum was analyzed at a potential of −48 mV, the absorption peak was at 557.5 nm. Thus, the high-potential species is probably cytochrome b_{558}, as would be predicted from the scheme in Fig. 5. It is unclear from the oxidation-reduction titrations of Pudek and Bragg (19) and Hendler et al. (12) whether cytochrome *o* was observed; however, cytochrome *d* was, apparently, not fully oxidized even at +400 mV, and, possibly, under these conditions, cytochrome *o* would not be oxidized.

Haddock et al. (10) observed that in aerobically grown cells, only two kinetically distinguishable *b*-type cytochromes were oxidized. According to the scheme proposed in Fig. 5, the rapidly oxidized species would consist of two components, cytochromes *o* and b_{558}, both of which could have been oxidized within the time resolution of the stopped-flow spectrophotometer (8). The slow-reacting species (with a half-time of oxidation of about 30 ms) may consist of the two cytochromes b_{556} and b_{562}, which could be difficult to resolve kinetically.

The electron transport sequence, as outlined in Fig. 5, can be arranged (Fig. 6) to take the same form as the carriers in the Mitchell "protonmotive ubiquinone cycle" (16). To make the sequence of carriers more obvious, the ubisemiquinone is depicted as diffusing across the membrane. However, as discussed by Mitchell (16), the ubisemiquinone may not need to diffuse across the membrane but may be reduced or oxidized at the other quinone reaction site on that side of the membrane at which the semi-

FIG. 6. *Electron transport chain of E. coli arranged in the form of the Mitchell proton motive ubiquinone cycle. Abbreviations: cyt, cytochrome; SH_2, reduced substrate; S, oxidized substrate; QH_2, ubiquinol; $QH^{\bullet}$, ubisemiquinone; Q, ubiquinone;* [Q]*, ubiquinone reaction center (see the text); e, electron.*

quinone is formed. There would be two protons translocated per electron transferred from NADH to oxygen and one proton translocated per electron transferred from D-lactate to oxygen. It is implied that proton translocation equivalent to site 1 of the mitochondrial system does not normally occur in *E. coli*. The points of interaction between the ubiquinone species and electron-donating components of the chain occur on the inner side of the membrane, and the points of interaction between the ubiquinone species and the electron-accepting components occur on the outer side of the membrane. The points of interaction are schematically represented by [Q] (Fig. 6). The proposed nature of the quinone species involved is speculative. A semiquinone-iron complex was previously proposed (7), and the retention of this species in the present scheme would require the availability of a diffusible form of iron within the membrane, such as, for example, the nonionic form of ferric chloride. The arrangement of the cytochromes across the membrane, but not their sequence, is also speculative, but it is hoped that the scheme proposed will provide a meaningful basis for further experimentation.

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